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# Protein Glycosylation Modification in Pichia Pastoris

### Field of the Invention

modifying the glycosylation process in methylotrophic yeast strains for the purpose of The present invention relates to methods and vectors useful for genetically producing glycoproteins with reduced glycosylation. The present invention further vectors, as well as glycoproteins produced from such genetically modified strains. relates to methylotrophic yeast strains generated using the present methods and S

#### Background of the Invention 9

The methylotrophic yeasts including Pichia pastoris have been widely used However, production and medical applications of some therapeutic glycoproteins can be hampered by the differences in the protein-linked carbohydrate biosynthesis for production of recombinant proteins of commercial or medical importance. between these yeasts and the target organism such as a mammalian subject.

where an N-linked oligosaccharide (Glo, Man, GlcNAcz) assembled on dolichol (a lipid an event common to all eukaryotic N-linked glycoproteins. The three glucose residues carrier intermediate) is transferred to the appropriate Asn of a nascent protein. This is and one specific  $\alpha$ -1,2-linked mannose residue are removed by specific glucosidases MangGloNAcs. The protein with this core sugar structure is transported to the Golgi and an  $\alpha$ -1,2-mannosidase in the ER, resulting in the core oligosaccharide structure, significant differences in the modifications of the sugar chain in the Golgi apparatus Protein N-glycosylation originates in the endoplasmic reticulum (ER), apparatus where the sugar moiety undergoes various modifications. There are between yeast and higher eukaryotes.

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different pathways depending on the protein moiety to which it is added. That is, (1) the core sugar chain does not change; (2) the core sugar chain is changed by adding In mammalian cells, the modification of the sugar chain proceeds via 3 the N-acetylglucosamine-1-phosphate moiety (GlcNAc-1-P) in UDP-N-acetyl

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(54) Title: PROTIEN GLYCOSYLATION MODIFICATION IN PICHIA PASTORIS

(55) Title: PROTIEN GLYCOSYLATION MODIFICATION IN PICHIA PASTORIS

(57) Abstract: The present invention provides genetically engineered strains of Pichia capable of producing proteins with reduced glycosylation. In particular, the genetically engineered strains of the present invention are capable of expressing either or both of the notes. L.2. manoidides and the decided such genetically engineered strains of the present invention can be further modified such status of Puchia are also provided. glycoxylation. In particular, the genetically engineered strains of the present invention are capable of expressing either or both of an ch.12-mamodides and glucosidate II. The genetically engineered strains of the present invention can be further modified such that the OCHI gene is disrupted. Methods of producing glycoxyrations with reduced glycoxylation using such genetically engineered stains of Pichia are also provided.

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glucosamine (UDP-GicNAc) to the 6-position of mannose in the core sugar chain, followed by removing the GicNAc moiety to form an acidic sugar chain in the glycoprotein; or (3) the core sugar chain is first converted into Man<sub>2</sub>GicNAc<sub>2</sub> by removing 3 mannose residues with mannosidase I; Man<sub>2</sub>GicNAc<sub>2</sub> is further modified

- 5 by adding GlcNAc and removing 2 more mannose residues, followed by sequentially adding GlcNAc, galactose (Gal), and N-acetylneuraminic acid (also called sialic acid (NeuNAc)) to form various hybrid or complex sugar chains (R. Komfeld and S. Komfeld, Ann. Rev. Biochem. 54: 631-664, 1985; Chiba et al J. Biol. Chem. 273: 26298-26304, 1998).
- In yeast, the modification of the sugar chain in the Golgi involves a series of additions of mannose residues by different mannosyltransferases ("outer chain" glycosylation). The structure of the outer chain glycosylation is specific to the organisms, typically with more than 50 mannose residues in *S. cerevistae*, and most commonly with structures smaller than Man<sub>14</sub>GlcNAc<sub>2</sub> in *Pichia pastoris*. This yeast-specific outer chain glycosylation of the high mannose type is also denoted hyperglycosylation.
- Hyperglycosylation is often undesired since it leads to heterogeneity of a recombinant protein product in both carbohydrate composition and molecular weight, which may complicate the protein purification. The specific activity (units/weight) of hyperglycosylated enzymes may be lowered by the increased portion of carbohydrate. In addition, the outer chain glycosylation is strongly immunogenic which is undesirable in a therapeutic application. Moreover, the large outer chain sugar can mask the immunogenic determinants of a therapeutic protein. For example, the influenza neuraminidase (NA) expressed in *P. pastoris* is glycosylated with N-glycans containing up to 30-40 mannose residues. The hyperglycosylated NA has a reduced immunogenicity in mice, as the variable and immunodominant surface loops on top of the NA molecule are masked by the N-glycans (Martinet et al. *Eur J. Biochem.* 247:

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332-338, 1997)

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Therefore, it is desirable to genetically engineer methylotrophic yeast strains in which glycosylation of proteins can be manipulated and from which recombinant proteins can be produced that would not be compromised in structure or function by large N-glycan side chains.

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### Summary of the Invention

The present invention is directed to methods and vectors useful for genetically modifying the glycosylation process in methylotrophic yeast strains to produce glycoproteins with reduced glycosylation. Methylotrophic yeast strains generated using the present methods and vectors, as well as glycoproteins produced from such genetically modified strains are also provided.

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In one embodiment, the present invention provides vectors useful for making genetically engineered methylotrophic yeast strains which are capable of producing glycoproteins with reduced glycosylation.

In one aspect, the present invention provides "knock-in" vectors which are capable of expressing in a methylotrophic yeast strain one or more proteins whose enzymatic activities lead to a reduction of glycosylation in glycoproteins produced by the methylotrophic yeast strain.

In a preferred embodiment, the knock-in vectors of the present invention include a nucleotide sequence coding for an  $\alpha$ -1,2-mannosidase or a functional part thereof and are capable of expressing the  $\alpha$ -1,2-mannosidase or the functional part in a methylotrophic yeast strain. A preferred nucleotide sequence is a nucleotide sequence encoding the  $\alpha$ -1,2-mannosidase of a fungal species, and more preferably,

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25 engineered such that the α-1,2-mannosidase or a functional part thereof expressed from the vector includes an ER-retention signal. A preferred ER-retention signal is HDEL. The α-1,2-mannosidase coding sequence can be operable linked to a constitutive or inducible promoter, and a 3' termination sequence. The vectors can be

Trichoderma reasei. Preferably, the a-1,2-mannosidase expression vector is

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integrative vectors or replicative vectors. Particularly preferred  $\alpha$ -1,2-mannosidase expression vectors include pGAPZMFManHDEL, pGAPZMFManMycHDEL, pPICZBMFManMycHDEL, pGAPZmManHDEL, pGAPZmMycManHDEL, pPIC9mMycManHDEL and pGAPZmMycManHDEL.

In another preferred embodiment, the knock-in vectors of the present invention include a sequence coding for a glucosidase II or a functional part thereof and are capable of expressing the glucosidase II or the functional part in a methylotrophic yeast strain. A preferred nucleotide sequence is a nucleotide sequence encoding the glucosidase II of a fungal species, and more preferably, Saccharomyces cerevisiae. Preferably, the glucosidase II expression vector is engineered such that the glucosidase II or a functional part thereof expressed from the vector includes an ER-retention signal. A preferred ER-retention signal is HDEL. The glucosidase II coding sequence can be operable linked to a constitutive or inducible promoter, and a 3' termination sequence. The vectors can be integrative vectors or replicative vectors. Particularly preferred glucosidase II expression vectors include pGAPZAGISII, pPICADEgISII, pPPICADEgISII, pPAOXZAGISII, pAOXZZAGISII, pGAPZAgISIIHDEL and pGAPADEgISII, pPICADEgISII, pAOXZADEGISII, pGAPZAgISIIHDEL and pGAPADEgISIIHDEL.

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- Expression vectors which include both of an  $\alpha$ -1,2-mannosidase expression unit and a glucosidase II expression unit are also provided by the present invention.
- In another aspect, the present invention provides "knock-out" vectors
  which, when introduced into a methylotrophic yeast strain, inactivate or disrupt a gene
  thereby facilitating the reduction in the glycosylation of glycoproteins produced in the
  methylotrophic yeast strain.

In one embodiment, the present invention provides a "Knock-out" vector which, when introduced into a methylotrophic yeast strain, inactivates or disrupts the Ochl gene. A preferred Ochl knock-out vector is pBLURA5'PpOCHI.

Still another embodiment of the present invention provides vectors which include both a knock-in unit and a knock-out unit.

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Furthermore, any of the knock-in or knock-out vectors of the present invention can also include a nucleotide sequence capable of expressing a heterologous protein of interest in a methylotrophic yeast.

Another embodiment of the present invention provides methods of modifying the glycosylation in a methylotrophic yeast by transforming the yeast with one or more vectors of the present invention.

Strains of a methylotrophic yeast which can be modified using the present methods include, but are not limited to, yeast strains capable of growth on methanol such as yeasts of the genera Candida, Hansenula, Torulopsis, and Pichia. Preferred methylotrophic yeasts are of the genus Pichia. Especially preferred are Pichia pastoris strains GS115 (NRRL Y-1881), GS190 (NRRL Y-18014), PPF1 (NRRL Y-18017), PPY120H, yGC4, and strains derived therefrom. Methylotrophic yeast strains which can be modified using the present methods also include those methylotrophic yeast strains which have been engineered to express one or more heterologous proteins

of interest. The glycosylation on the heterologous proteins expressed from these previously genetically engineered strains can be reduced by transforming such strains with one or more of the vectors of the present invention

Methylotrophic yeast strains which are modified by practicing the present methods are provided in another embodiment of the present invention.

A further aspect of the present invention is directed to methods of producing glycoproteins with a reduced glycosylation.

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In accordance with such methods, a nucleotide sequence capable of expressing a glycoprotein can be introduced into a methylotrophic yeast strain which has previously been transformed with one or more of the vectors of the present

invention. Alternatively, a methylotrophic yeast strain which has been genetically engineered to express a glycoprotein can be transformed with one or more of the vectors of the present invention. Moreover, if a methylotrophic yeast strain is not transformed with a nucleotide sequence encoding a glycoprotein of interest or any of the vectors of the present invention, such yeast strain can be transformed, either

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consecutively or simultaneously, with both a nucleotide sequence capable of expressing the glycoprotein and one or more vectors of the present invention.

Additionally, a methylotrophic yeast strain can be transformed with one or more of the present knock-in and/or knock-out vectors which also include a nucleotide sequence capable of expressing a glycoprotein in the methylotrophic yeast strain.

Glycoproteins products produced by using the methods of the present invention, i.e., glycoproteins with reduced N-glycosylation, are also part of the present invarious

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Kits which include one or more of the vectors of the present invention, or one or more strains modified to produce glycoproteins with reduced glycosylation, are also provided.

## Brief Description of the Drawings

Figure 1 depicts vectors carrying an HDEL-tagged *Trichoderma reesel* or 1,2-mannosidase expression cassette and describes the way in which these vectors were constructed according to methods known in the art. Abbreviations used throughout construction schemes: 5' AOX1 or AOX1 P: *Pichia pastoris* AOX1 promoter sequence; Amp R: ampicillin resistance gene; ColE1: ColE1 origin of replication; 3' AOX1: 3' sequences of the *Pichia pastoris* AOX1 gene; HIS4: HIS4 gene of *Pichia pastoris*. AOXT T: transcription terminator sequence of the *Pichia pastoris* AOX1 gene; ORF: open reading frame; S: secretion signal; P TEF1: the promoter sequence of the *Saccharomyces cerevisiae* transcription elongation factor 1

25 sequence of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene;
PpURA3: *Pichia* pastoris URA3 gene. As can be seen in this figure, the *Trichoderma*reesel a-1,2-mannosidase was operably linked to the coding sequence for the S.
cerevisiae a-mating factor secretion signal sequence and further operably linked at the
3' terminus of the coding sequence to the coding sequence for an HDEL peptide. The

resistance gene; CYC1 TT: 3' end of the S. cerevisiae CYC1 gene; GAP: promoter

gene; P EM7: synthetic constitutive prokaryotic promotor EM7; Zeocin: Zeocin

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whole fusion construct was operably linked to either the P. pastoris AOX1 promoter (in pPIC9MFManHDEL) or to the P. pastoris GAP promotor (in pGAPZMFManHDEL).

mannosidase IB expression cassette and describes the way in which these vectors were sequence and the coding sequence for the catalytic domain of the Mus musculus α-1,2cassette were made in which the coding sequence for a cMyc epitope tag was inserted Figure 2 depicts vectors carrying an HDEL-tagged Mus musculus a-1,2constructed according to methods known in the art. As can be seen in this figure, the catalytic domain of the Mus musculus a-1,2-mannosidase IB was operably linked to the coding sequence for the S. cerevistae a-mating factor secretion signal sequence sequence for an HDEL peptide. The whole fusion construct was operably linked to either the P. pastoris AOX1 promoter (in pPIC9mManHDEL) or to the P. pastoris between the coding sequence for the S. cerevisiae a-Mating Factor secretion signal and further operably linked at the 3' terminus of the coding sequence to the coding mannosidase IB. This expression cassette was also operably linked to either the P. GAP promotor (in pGAPZmManHDEL). Furthermore, variants of the expression pastoris AOX1 promoter (in pPIC9mMycManHDEL) or to the P. pastoris GAP promotor (in pGAPZmMycManHDEL). S 2 15

Figure 3 depicts vectors carrying a MycHDEL tagged Thichoderma reesel o-1,2-mannosidase and the way in which these vectors were obtained. The resulting fusion construction was again operably linked to either the P. pastoris AOX1 promoter (in pPICZBMFManMycHDEL) or to the P. pastoris GAP promotor (in pGAPZMFManMycHDEL).

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Figure 4 demonstrates the intracellular localization of the MycHDEL-tagged Trichoderma reeset α-1,2-mannosidase and indicates ER-targeting by immunofluorescence analysis. Panel A Western blotting. Yeast strains were grown in 10 ml YPG cultures to an OD<sub>800</sub>=10, diluted fivefold and grown in YPM for 48 h.

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10 ml YPG cultures to an OD<sub>600</sub>=10, diluted fivefold and grown in YPM for 48 h. 1/50th of the culture medium and 1/65th of the cells were analysed by SDS-PAGE and

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lysates. 1,2: pGAPZMFManMycHDEL transformants. 3: non-transformed PPY12OH Western blotting with the mouse monoclonal 9E10 anti-Myc antibody. The position of molecular weight marker proteins are indicated with arrows. Lanes 1-5: cellular (negative control). 4,5: pPICZBMFManMycHDEL transformants. Lanes 6-10:

- a P. pastoris cell (strain PPY12OH transformed with pGAPZMFManHDEL) at 1000x transformants. Panel B Immunofluorescence microscopy. 1: phase contrast image of magnification. The nucleus is visible as an ellipse in the lower right quadrant of the pGAPZMFManMycHDEL transformants. 9,10: pPICZBMFManMycHDEL culture media. 6: non transformed PPV12OH (negative control). 7,8: Ś
- cell. 2: same cell as in 1, but in fluorescence microscopy mode to show localization of the T. reeset mannosidase-Myc-HDEL protein. The protein is mainly localized in a distribution throughout the cytoplasm, with 3-4 dots per cell is typical for cis-Golgi circular distribution around the nucleus (nuclear envelope), which is typical for an magnification. 4: same cell in fluorescence microscopy to show localization of the pastoris cell (strain PPY120H transformed with pGAPZMFManHDEL) at 1000x endoplasmic reticulum steady-state distribution. 3: phase contrast image of a P. Golgi marker protein OCH1-HA in P. pastoris strain PPY12OH. The dot-like 2
  - distribution in P. pastoris. 2
- Protein Disulfide Isomerase in sucrose density gradient centrifugation. The top panel bottom panel shows the distribution of the MycHDEL-tagged Trichoderma reesei a-Figure 5 depicts the co-sedimentation of mannosidase-MycHDEL with MycHDEL almost exactly matches the distribution of the ER marker PDI and thus OCHI-HA Golgi marker protein. The middle panel shows this distribution for the Protein Disulfide Isomerase endoplasmic reticulum marker protein. Finally, the shows the distribution over the different fractions of the sucrose gradient of the 1,2-mannosidase over the same fractions. It is concluded that the mannosidasemainly resides in the ER of the Pichia pastoris yeast cells.

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sialidase coexpressed with Trichoderma reesel mannosidase-HDEL. Panel A: malto-Figure 6 depicts the N-glycan analysis of Trypanosoma cruzi trans-

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cruzi trans-sialidase expressed in Pichia pastoris. The peak at GU=9,2 corresponds to malto-oligosaccharides. Panel B: N-glycans derived from recombinant Trypanosoma oligosaccharide size reference ladder. Sizes of the glycans are expressed in Glucose Units (GU) by comparison of their electrophoretic mobility to the mobility of these

- Man<sub>8</sub>GlcNAc<sub>2</sub>. Panel C: same analytes as panel 2, but after overnight treatment with GU=7,6 corresponds to the Man<sub>5</sub>GlcNAc<sub>2</sub> peak in the profile of RNase B (Panel F). derived from recombinant trans-sialidase co-expressed in Pichia pastoris with T. reesei mannosidase-HDEL (under control of the GAP promotor). The peak at 3U/ml purified recombinant T. reeset a-1,2-mannosidase. Panel D: N-glycans Ś
- bovine RNase B. These glycans consist of MansGlcNAc2 to MansGlcNAc2. Different purified recombinant T. reeset α-1,2-mannosidase. Panel F: N-glycans derived from Panel E: same analytes as panel D, but after overnight treatment with 3 mU/ml isomers are resolved, accounting for the number of peaks for Man<sub>7</sub>GlcNAc<sub>2</sub>. 2

HDEL-tagged Trichoderma reesei a-1,2-mannosidase and the HDEL-tagged catalytic Figure 7 depicts the processing of influenza haemagglutinin N-glycans by oligosaccharide size reference ladder. Panel 2: N-glycans derived from recombinant oligosaccharide runs at scan 1850 in this analysis (not shown). Panel 1: maltoinfluenza haemagglutinin expressed in Pichia pastoris. The peak at scan 2250 domain of murine α-1,2-mannosidase IB. The Man<sub>5</sub>GlcNAc<sub>2</sub> reference 12

- with 30 mU purified recombinant T. reeset a-1,2-mannosidase. Panel 5: N-glycans MangGlcNAcy. Panel 4: Same analytes as for panel 3, but after overnight treatment haemagglutinin co-expressed in Pichia pastoris with T. reeset mannosidase-HDEL corresponds to ManyGlcNAc2. Panel 3: N-glycans derived from recombinant (under control of the GAP promotor). The peak at scan 1950 corresponds to 8
- as for panel 5, but after overnight treatment with 30 mU purified recombinant  $\it T.$  reesei derived from recombinant haemagglutinin co-expressed in Pichia pastoris with mouse mannosidase IB-HDEL (under control of the GAP promotor). Panel 6: same analytes 25

Figure 8 graphically depicts vector pBLURAS'PpOCH1 and the way in which it was constructed.

Pigure 9 depicts the scheme for disrupting the *Pichta pastoris* OCH1 gene by single homologous recombination using pBLURA5'PpOCH1.

Figure 10 depicts the cell wall glycoprotein N-glycan analysis of the Ochl-inactivated clone and three clones derived from this Ochl-inactivated clone by transformation with pGAPZMFManHDEL. Panel 1 shows the analysis of a mixture of malto-oligosaccharides, the degree of polymerisation of which is given by the numbers on the very top of the figure. This analysis serves as a size reference for the

other panels. On the vertical axis of all panels, peak intensity in relative fluorescence units is given. Panel 2-6: analysis of the cell wall glycoprotein N-glycans of the following strains: Panel 2, non-engineered P. pastorts strain yGC4; Panel 3, yGC4 transformed with pBLURASPpOch1; 4-6, three clones of the strain of Panel 3, supplementarily transformed with pGAPZMFManHDEL. Panel 7: the N-glycans derived from bovine RNaseB, consisting of a mixture of Man<sub>5-9</sub>GlcNAc<sub>2</sub>. As can be seen from comparison between panel 2 and 3 and reference to panel 7, transformation with pBLURASPpOch1 leads to a strongly increased abundance of the Man<sub>5</sub>GlcNAc

seen from comparison between panel 2 and 3 and reference to panel 7, transformation with pBLURA5PpOch1 leads to a strongly increased abundance of the Man<sub>2</sub>GlcNAc<sub>2</sub> substrate N-glycan (named peak 1 in Panel 2) of OCH1p. Peak 2 represents the Man<sub>3</sub>GlcNAc<sub>2</sub> product of OCH1p. Furthermore, upon supplementary transformation of pGAPZMFManHDEL, the major glycan on the cell wall glycoproteins of three independent clones is the Man<sub>3</sub>GlcNAc<sub>2</sub> end product (peak 3 in panel 4) of T. reesel oc-1,2-mannosidase digestion of the Man<sub>3</sub>GlcNAc<sub>2</sub> substrate.

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Figure 11 depicts the analysis of exactly the same glycan mixtures as in Figure 10, but after an *in vitro* digest with 3mU/ml purified *Trichoderma reesei* α-1,2-mannosidase, overnight in 20 mlM sodium acetate pH=5.0. Axis assignment is the same as in Figure 10. More Man<sub>3</sub>GlcNAc<sub>2</sub> is formed in the pBLURA5'PpOch1 transformed strain (Panel 3) than in the parent strain (Panel 2). Peaks in all panels before scan 3900 come from contaminants and should be ignored in the analysis.

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Figure 12 depicts the expression vector pGAPZAGLSII (SEQ ID NO: 18). P TEF1: promotor of S. cerevisiae transcription elongation factor gene. P Em7:

synthetic prokaryotic promotor. Zeocin: zeocine resistance marker gene. CYC1 TT: transcription terminator of S. cerevisiae cytochrome CI gene. Col BI: bacterial origin

5 of replication. GAP: promotor of the P. pasttoris GAP gene. GLS2: S. cerevisiae glucosidase II gene. AOX1 TT: transcription terminator of the P. pastoris AOX1 gene

Figure 13 depicts the expression vector pAOX2ZAGLSII (SEQ ID NO:

P TEF1: promotor of S. cerevisiae transcription elongation factor gene. P Em7:
 synthetic prokaryotic promotor. Zeocin: zeocine resistance marker gene. CYC1 TT: transcription terminator of S. cerevisiae cytochrome CI gene. Col B1: bacterial origin of replication. AOX2 P: promotor of the P. pastoris AOX2 gene. GLS2: S. cerevisiae glucosidase II gene. AOX1 TT: transcription terminator of the P. pastoris AOX1 gene

Figure 14 depicts the expression vector pPICZAGLSII (SEQ ID NO: 20).

15 P TEF1: promotor of S. cerevisiae transcription elongation factor gene. P Em7: synthetic prokaryotic promotor. Zeocin: zeocine resistance marker gene. CYC1 TT: transcription terminator of S. cerevisiae cytochrome Cl gene. Col El: origin of replication. AOXI P: promotor of the P. pastoris AOXI gene. GLS2: S. cerevisiae glucosidase Il gene. AOXI TT: transcription terminator of the P. pastoris AOXI gene

Figure 15 depicts the expression vector pVPT1ZAGLSII ((SEQ ID NO: 22). P TEF1: promotor of S. cerevisiae transcription elongation factor gene. P Em7: synthetic prokaryotic promotor. Zeocin: zeocine resistance marker gene. CYC1 TT: transcription terminator of S. cerevisiae cytochrome CI gene. Col EI: origin of replication. P YPT1: promotor of the P. pastoris YPTI gene. GLS2: S. cerevisiae

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25 glucosidase II gene. AOX1 TT: transcription terminator of the P. pastoris AOX1 gene.

Figure 16 depicts the expression vector pGAPADE1gIsII (SEQ ID NO: 19). Amp R: Ampillicin resistance marker gene. ADE1: P. pastoris ADE1 selection

glucosidase II gene. AOX1 TT: transcription terminator of the P. pastoris AOX1 gene marker gene. GAP: promotor of the P. Pastoris GAP gene. GLS2: S. cerevisiae

Figure 17 depicts the expression vector pAOX2ADE1gIsII (SEQ ID NO: 17). Amp R: Ampillicia resistance marker gene. ADE1: P. pastoris ADE1 selection

marker gene. AOX2 P: promotor of the P. pastoris AOX2 gene. GLS2: S. cerevisiae glucosidase II gene. AOX1 TT: transcription terminator of the P. pastoris AOX1 gene.

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Figure 18 depicts the expression vector pPICADE1glsII (SEQ ID NO: 21). Amp R: Ampillicin resistance marker gene. ADB1: P. pastoris ADB1 selection

marker gene. AOX1 P: promotor of the P. pastoris AOX1 gene. GLS2: S. cerevisiae glucosidase II gene. AOX1 TT: transcription terminator of the P. pastoris AOX1 2

23). Amp R: Ampillicin resistance marker gene. ADB1: P. pastoris ADE1 selection Figure 19 depicts the expression vector pYPT1ADE1glsII (SEQ ID NO: marker gene. P YPT1: promotor of the P. pastoris YPT1 gene. GLS2: S. cerevisiae glucosidase II gene. AOX1 TT: transcription terminator of the P. pastoris AOX1

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gene.

Figure 20 depicts the expression vector pGAPZAgIsIIHDEL (SEQ ID NO: 24). Amp R: Ampillicin resistance marker gene. ADE1: P. pastoris ADE1 selection glucosidase II gene. AOX1 TT: transcription terminator of the P. pastoris AOX1 marker gene. GAP: promotor of the P. pastoris GAP gene. GLS2: S. cerevisiae gene.

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Em7: synthetic prokaryotic promotor. Zeocin: zeocine resistance marker gene. CYC1 Figure 21 depicts the expression vector pGAPADE1 glsIIHDEL (SEQ ID TT: transcription terminator of S. cerevisiae cytochrome Cl gene. Col El: bacterial NO: 25). P TEF1: promotor of S. cerevisiae transcription elongation factor gene. P cerevisiae glucosidase II gene. AOX1 TT: transcription terminator of the P. pastoris origin of replication. GAP: promotor of the P. pastoris GAP gene. GLS2: S.

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4-methylumbellyferyl-alpha-D-glucopyranoside and alpha-glucosidase from Sigma. 1: Figure 22 depicts the test of the GLSII activity assay using a commercially contains phosphate-citrate buffer pH 6.8, mannose, 2-deoxy-D-glucose, the substrate available yeast alpha-glucosidase (Sigma: Cat. No. G-5003). The assay mixture

as 1, but this time, the assay mixture lacks the alpha-glucosidase; 3: same as 1, but this assay mixture illuminated with UV-light after overnight incubation at 37 ° C; 2: same time, the assay mixture lacks the substrate. S

(Sigma: Cat. No. G-5003); 2: assay with the purified medium of strain 18 (PPY12-OH transformed with pGAPZAGLSII); 3: assay with purified medium of the WT PPY12-OH strain; 4: assay with the purified medium of strain H3 (PPY12-OH transformed Figure 23 depicts the results of the activity of recombinantly expressed GLSII from Pichia pastoris. All assay mixtures were incubated overnight at 37 °C and afterwards illuminated with UV-light. 1: assay with yeast alpha-glucosidase with pGAPZAgIsIIHDEL). 2

Detailed Description of the Invention

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chains. Such hyperglycosylation of recombinant glycoproteins is undesirable in many oligosaccharide structure. After the proteins are transported from the ER to the Golgi different mannosyltransferases, resulting in glycoproteins with large mannose side It has been established that the majority of N-glycans on glycoproteins leaving the endoplasmic reticulum (ER) of Pichia have the core MangGlcNAc2 apparatus, additional mannose residues are added to this core sugar moiety by

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reduced glycosylation. Methylotrophic yeast strains generated using the present methods and vectors, as well as glycoproteins produced from such genetically modified strains are also provided. ង

genetically modifying methylotrophic yeast strains to produce glycoproteins with

instances. Accordingly, the present invention provides methods and vectors for

genetically modifying methylotrophic yeast strains to produce glycoproteins with In one embodiment, the present invention provides vectors useful for reduced glycosylation In one aspect, the present invention provides "knock-in" vectors which are enzymatic activities lead to a reduction of glycosylation in glycoproteins produced by capable of expressing in a methylotrophic yeast strain one or more proteins whose the methylotrophic yeast strain. According to the present invention, such proteins include, e.g., an a-1,2-mannosidase, a glucosidase II, or functional parts thereof.

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sequence coding for an a-1,2-mannosidase or a functional part thereof and are capable of expressing the  $\alpha$ -1,2-mannosidase or the functional part in a methylotrophic yeast In a preferred embodiment, the vectors of the present invention include a strain.

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any Pichia Golgi mannosyltransferase, i.e., mannose residues can not be added to this mammalian N-acetylglucosaminyl-transferase I and is an intermediate for the hybridand complex-type sugar chains characteristic of mammalian glycoproteins. Thus, by way of introducing an a-1,2-mannosidase into methylotrophic yeasts such as Pichia, glycoproteins to Man<sub>5</sub>GlcNAc<sub>2</sub>. In vitro, Man<sub>5</sub>GlcNAc<sub>2</sub> is a very poor substrate for An  $\alpha$ -1,2-mannosidase cleaves the  $\alpha$ -1,2-linked mannose residues at the sugar structure. On the other hand, MansGlcNAc2 is the acceptor substrate for the non-reducing ends of MangGicNAc, and converts this core oligosaccharide on glycoproteins with reduced mannose content can be produced.

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According to the present invention, the nucleotide sequence encoding an  $\alpha$ a-1,2-mannosidase (Tremblay et al. Glycobiology 8: 585-595, 1998), as well as fungal rabbit a-1,2-mannosidase (Lal et al. J. Biol. Chem. 269: 9872-9881, 1994) or a human murine α-1,2-mannosidase (Herscovics et al. J. Biol. Chem. 269: 9864-9871, 1994), a 1,2-mannosidase for use in the expression vector of the present invention can derive from any species. A number of a-1,2-mannosidase genes have been cloned and are available to those skilled in the art, including mammalian genes encoding, e.g., a

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Saccharomyces cerevisiae a-1,2-mannosidase. Protein sequence analysis has revealed a high degree of conservation among the eukaryotic a-1,2-mannosidases identified so genes encoding, e.g., an Aspergillus a-1,2-mannosidase (msdS gene), a Trichoderma reesei a-1,2-mannosidase (Maras et al. J. Biotechnol. 77: 255-263, 2000), or a far. Preferably, the nucleotide sequence for use in the present vectors encodes a fungal  $\alpha$ -1,2-mannosidase, more preferably, a Trichoderma reesel  $\alpha$ -1,2-mannosidase, and more particularly, the Trichoderma reesel a-1,2-mannosidase described by Maras et al. J. Biotechnol. 77: 255-63 (2000).

According to the present invention, the nucleotide sequence can also code for only a functional part of an  $\alpha$ -1,2-mannosidase. 2

B. Those skilled in the art can readily identify and make functional parts of an α-1,2protein. By "substantially" is meant at least about 40%, or preferably, at least 50% or example, as illustrated by the present invention, the catalytic domain of the murine  $\alpha$ more of the enzymatic activity of the full-length a-1,2-mannosidase is retained. For 1,2-mannosidase IB constitutes a "functional part" of the murine  $\alpha$ -1,2-mannosidase mannosidase using a combination of techniques known in the art. Predictions of the mannosidase which substantially retains the enzymatic activity of the full-length By "functional part" is meant a polypeptide fragment of an  $\alpha$ -1,2-12

portions of an  $\alpha$ -1,2-mannosidase essential to or sufficient to confer the enzymatic activity can be made based on analysis of the protein sequence. The activity of a appropriate expression system, can be verified using in vitro or in vivo assays portion of an \alpha-1,2-mannosidase of interest, expressed and purified from an described hereinbelow. 2

functional part thereof expressed in a methylotrophic yeast strain preferably is targeted In accordance with the present invention, an α-1,2-mannosidase or a to a site in the secretory pathway where Man<sub>8</sub>GlcNAc<sub>2</sub> (the substrate of  $\alpha$ -1,2-22

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glycosyltransferase which elongates the sugar chain with additional mannose residues. mannosidase) is already formed on a glycoprotein, but has not reached a Golgi

or a functional part thereof expressed from the vector includes an ER-retention signal. 1,2-mannosidase expression vector is engineered as such that the \alpha-1,2-mannosidase Accordingly, in a preferred embodiment of the present invention, the  $\alpha$ -

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Such ER retention sequences are often found in proteins that reside and function in the "An ER retention signal" refers to a peptide sequence which directs a protein having such peptide sequence to be transported to and retained in the ER. 됬

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Multiple choices of ER retention signals are available to those skilled in the (Martinet et al. Biotechnology Letters 20: 1171-1177, 1998). A preferred ER retention signal for use in the present invention is peptide HDEL (SEQ ID NO: 1). The HDEL with an HDBL sequence are bound by a membrane-bound receptor (Erd2p) and then retention/retrieval signal for the ER (Pelham EMBO J. 7: 913-918, 1988). Proteins enter a retrograde transport pathway for return to the ER from the Golgi apparatus. peptide sequence, found in the C-terminus of a number of yeast proteins, acts as a art, e.g., the first 21 amino acid residues of the S. cerevisiae ER protein MNS1 2

According to the present invention, an ER retention signal can be placed anywhere in the protein sequence of an a-1,2-mannosidase, but preferably at the C-

terminus of the  $\alpha$ -1,2-mannosidase. ន

modified, e.g., by insertion of an epitope tag to which antibodies are available, such as The  $\alpha$ -1,2-mannosidase for use in the present invention can be further Myc, HA, FLAG and His6 tags well-known in the art. An epitope-tagged a-1,2mannosidase can be conveniently purified, or monitored for both expression and intracellular localization.

protein of interest by inserting nucleotide sequences coding for such signal or tag into An ER retention signal and an epitope tag can be readily introduced into a

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the nucleotide sequence encoding the protein of interest, using any of the molecular biology techniques known in the art.

capable of expressing the glucosidase II or the functional part in the methylotrophic include a sequence coding for a glucosidase II or a functional part thereof and are In another preferred embodiment, the vectors of the present invention S

specific glucosidases to remove the glucose residues, and by a mannosidase to remove one specific a-1,2-linked mannose. It has been observed by the present inventors that glucose molecules present on the sugar structure prevent the complete digestion of the some recombinant proteins expressed in Pichia have residual glucose residues on the sugar moiety when such proteins leave the ER for the Golgi apparatus. The residual (Glc3Man,GlcNAc2), transferred in the ER onto a protein, is cleaved in the ER by sugar moiety by an α-1,2-mannosidase, and the introduction of an exogenous It has been established that the initial N-linked oligosaccharide

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glucosidase II protein from these mammalian species consists of an alpha and a beta glucosidase II can derive from any species. Glucosidase II genes have been cloned According to the present invention, the nucleotide sequence encoding a from a number of mammalian species including rat, mouse, pig and human. The

glucosidase can facilitate the removal of these glucose residues.

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gene from S. cerevisiae has also been cloned (ORP YBR229c, located on chromosome enzyme, while the beta subunit has a C-terminal HDEL ER-retention sequence and is subunit. The alpha subunit is about 110 kDa and contains the catalytic activity of the believed to be important for the ER localization of the enzyme. The glucosidase II 2

II). This gene encodes a protein of about 110 kDa, which shows a high degree of homology to the mammalian alpha subunits. 23

A preferred glucosidase II gene for use in the present vectors is from a fungal species such as Pichia pastoris and S. cerevisiae. An example of a fungal glucosidase II gene is the S. cerevisiae glucosidase II alpha subunit gene.

According to the present invantion, the nucleotide sequence can also encode only a functional part of a glucosidase II. By "functional part" is meant a polypeptide fragment of a glucosidase II which substantially retains the enzymatic activity of the full-length protein. By "substantially" is meant at least about 40%, or preferably, at least 50% or more of the enzymatic activity of the full-length

made by those skilled in the art using a variety of techniques known in the art.

In a preferred embodiment of the present invention, the glucosidase II protein is engineered to include an RR retention signal such that the protein expressed

glucosidase II is retained. Functional parts of a glucosidase II can be identified and

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in a methylotrophic yeast strain is targeted to the ER and retains therein for function.

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ER retention signals are as described hereinabove, e.g., the HDEL peptide sequence.

The glucosidase II for use in the present invention can be further modified, e.g., by insertion of an epitope tag to which antibodies are available, such as Myc, HA, FLAG, and His6 tag, which are well-known in the art.

15 According to the present invention, the "knock-in" vectors can include either or both of an α-1,2-mannosidase coding sequence and a glucosidase II coding sequence.

Further according to the present invention, the nucleotide sequence coding for the enzyme to be expressed (e.g., an  $\alpha$ -1,2-mannosidase or a functional part thereof, or a glucosidase II or a functional part thereof) can be placed in an operable linkage to a promoter and a 3' termination sequence.

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Promoters appropriate for expression of a protein in a methylotrophic yeast can include both constitutive promoters and inducible promoters. Constitutive promoters include e.g., the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase promoter ("the GAP promoter"). Examples of inducible promoters include, e.g., the *Pichia pastoris* alcohol oxidase I promoter ("the AOXI promoter") (U.S. Patent No. 4,855,231), or the *Pichia pastoris* formaldehyde dehydrogenase promoter ("the FLD promoter") (Shen et al. Gene 216: 93-102, 1998).

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3' termination sequences are sequences 3' to the stop codon of a structural gene which function to stabilize the mRNA transcription product of the gene to which the sequence is operably linked, such as sequences which elicit polyadarylation. 3' termination sequences can be obtained from Pichia or other methylotrophic yeast.

5 Examples of Pichia pastoris 3' termination sequences useful for the practice of the present invention include termination sequences from the AOXI gene, p40 gene, HIS4 gene and FLDI gene.

The vectors of the present invention preferably contain a selectable marker gene. The selectable marker may be any gene which confers a selectable phenotype upon a methylotrophic yeast strain and allows transformed cells to be identified and selected from untransformed cells. The selectable marker system may include an auxotrophic mutant methylotrophic yeast strain and a wild type gene which complements the host's defect. Examples of such systems include the Saccharomyces cerevisiae or Pichia pastoris HIS4 gene which may be used to complement his4

15 Pichia strains, or the S. cerevisiae or Pichia pastoris ARG4 gene which may be used to complement Pichia pastoris arg mutants. Other selectable marker genes which function in Pichia pastoris include the Zeo<sup>R</sup> gene, the G418<sup>R</sup> gene, and the like.

The vectors of the present invention can also include an autonomous replication sequence (ARS). For example, U.S. Patent No. 4,837,148 describes autonomous replication sequences which provide a suitable means for maintaining plasmids in *Plehta pastoris*. The disclosure of U.S. Patent No. 4,837,148 is incorporated herein by reference.

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The vectors can also contain selectable marker genes which function in bacteria, as well as sequences responsible for replication and extrachromosomal maintenance in bacteria. Examples of bacterial selectable marker genes include ampicillin resistance (Amp), tetracycline resistance (Tet), noomycin resistance, hygromycin resistance, and zoocin resistance (Zeo<sup>6</sup>) genes.

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According to the present invention, the nucleotide sequence encoding the protein to be expressed in a methylotrophic yeast can be placed in an integrative vector or a replicative vector (such as a replicating circular plasmid).

Integrative vectors are disclosed, e.g., in U.S. Patent No. 4,882,279 which is incorporated herein by reference. Integrative vectors generally include a serially arranged sequence of at least a first insertable DNA fragment, a selectable marker gene, and a second insertable DNA fragment. The first and second insertable DNA fragments are each about 200 nucleotides in length and have nucleotide sequences which are homologous to portions of the genomic DNA of the species to be

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10 transformed. A nucleotide sequence containing a structural gene of interest for expression is inserted in this vector between the first and second insertable DNA fragments whether before or after the marker gene. Integrative vectors can be linearized prior to yeast transformation to facilitate the integration of the nucleotide sequence of interest into the host cell genome.

15 Replicative and integrative vectors carrying either or both of an o-1,2mannosidase coding sequence or a glucosidase II coding sequence can be constructed
by standard techniques known to one of ordinary skill in the art and found, for
example, in Sambrook et al. (1989) in Molecular Cloning: A *Laboratory Manual*, or
any of a myriad of laboratory manuals on recombinant DNA technology that are
widely available.

Preferred vectors of the present invention carrying an α-1,2-mannosidase expression sequence include pGAPZMFManHDEL, pGAPZMFManMycHDEL, pPICZBMFManMycHDEL, pGAPZmManHDEL, pGAPZmMycManHDEL, pPIC9mMycManHDEL and pGAPZmMycManHDEL, which are further described in the Examples hereinbelow.

Preferred vectors of the present invention carrying a glucosidase II expression sequence include pGAPZAGLSII, pPICZAGLSII, pAOXZZAGLSII, pYPTIZAGLSII, pGAPADE1gisII, pPICADE1gisII, pAOXZADE1gisII,

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pYPTIADE1glsII, pGAPZAgIsIIHDEL and pGAPADE1glsIIHDEL, which are further described in the Examples hereinbelow.

In another aspect, the present invention provides "knock-out" vectors
which, when introduced into a methylotrophic yeast strain, inactivate or disrupt a gene
thereby facilitating the reduction in the glycosylation of glycoproteins produced in the
methylotrophic yeast strain.

In one embodiment, the present invention provides a "knock-out" vector which, when introduced into a methylotrophic yeast strain, inactivates or disrupts the Och1 gene.

10 The S. cerevisiae OCH1 gene has been cloned (Nakayama et al. EMBO J. 11: 2511-2519, 1992). It encodes a membrane bound α-1,6-mannosyltransferase, localized in the early Golgi complex, that is functional in the initiation of α-1,6-polymannose outer chain addition to the N-linked core oligosaccharide (Man<sub>3</sub>GlcNAc<sub>2</sub> and Man<sub>3</sub>GlcNAc<sub>3</sub>) (Nakanishi-Shindo et al. J. Blol. Chem. 268: 26338-26345, 1993).

A PIchia sequence has been described in Japanese Patent Application No. 07145005 that encodes a protein highly homologous to the S. cerevisiae OCH1. For purpose of the present invention, this sequence is denoted herein as "the Pichia OCH1 gene". Those skilled in the art can isolate the OCH1 genes from other methylotrophic yeasts using techniques well known in the art.

20 According to the present invention, a disruption in the OCH1 gene of a methylotrophic yeast can result in either the production of an inactive protein product or no product. The disruption may take the form of an insertion of a heterologous DNA sequence into the coding sequence and/or the deletion of some or all of the coding sequence. Gene disruptions can be generated by homologous recombination essentially as described by Rothstein (in *Methods in Enymology*, Wu et al., eds., vol 101:202-211, 1983).

To disrupt the Ochl gene by homologous recombination, an Ochl knockout vector can be constructed in such a way to include a selectable marker gene. The selectable marker gene is operably linked, at both 5' and 3' end, to portions of the

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Och1 gene of sufficient length to mediate homologous recombination. The selectable auxotrophy or provide antibiotic resistance, including URA3, LEU2 and HIS3 genes. marker can be one of any number of genes which either complement host cell Other suitable selectable markers include the CAT gene, which confers

- genome and the disruption of the Ochl gene can be determined based on the selection of an Och1 knock-out vector are then introduced into host methylotrophic yeast cells colonies due to the expression of active β-galactosidase. Linearized DNA fragments chloramphenicol resistance on yeast cells, or the lacZ gene, which results in blue using methods well known in the art. Integration of the linear fragments into the S
  - Alternatively, an Och1 knock-out vector can be constructed in such a way marker and can be verified by, for example, Southern Blot analysis. 2

to include a portion of the Och1 gene to be disrupted, which portion is devoid of any Och1 promoter sequence and encodes none or an inactive fragment of the Och1

- protein. By "an inactive fragment", it is meant a fragment of the Ochl protein which has, preferably, less than about 10% and most preferably, about 0% of the activity of the full-length OCH1 protein. Such portion of the OCH1 gene is inserted in a vector in such a way that no known promoter sequence is operably linked to the OCHI 12
- sequence, but that a stop codon and a transcription termination sequence are operably linked to the portion of the Ochl gene. This vector can be subsequently linearized in
- the portion of the OCH1 sequence and transformed into a methylotrophic yeast strain recombination, this linearized vector is then integrated in the OCH1 gene. Two Och1 sequences are produced in the chromosome as a result of the single homologous using any of the methods known in the art. By way of single homologous ន
- yeast, yet cannot produce an active OCH1 protein as such Och1 sequence codes for no known promoter sequence and thus, no active messenger is expected to be formed for vector, which is now under control of the OCH1 promoter of the host methylotrophic or an inactive fragment of the OCH1 protein, as described hereinabove. The second recombination. The first Och1 sequence is the portion of the Och1 gene from the Och1 sequence is a full OCH1 coding sequence, but is not operably linked to any ដ

introduced in the OCH1 sequence, to the 5' end of the site of linearization of the vector mutation" it is meant a mutation introducing a stop codon, a frameshift mutation or and to the 3' end of the translation initiation codon of OCH1. By "inactivating synthesis of an active OCH1 protein. Preferably, an inactivating mutation is

- gnown in the art. Such inactivating mutation ensures that no functional OCHI protein can be formed even if there exist some promoter sequences 5' to the Och1 sequence in introduced into an Och I sequence using any of the site directed mutagenesis methods any other mutation causing a disruption of the reading frame. Such mutation can be the knock-out vector.
- A preferred Och1 knock-out vector of the present invention is pBLURAS'PpOCHI.

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glucosidase expression sequence can be carried on the same plasmid used to disrupt If desired, either or both of a mannosidase expression sequence and a the OCH1 gene to create a "knock-in-and-knock-out" vector.

Additionally, any of the above-described vectors can further include a nucleotide sequence capable of expressing a glycoprotein of interest in a methylotrophic yeast strain.

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Another aspect of the present invention is directed to methods of modifying methylotrophic yeast strains. In accordance with the present methods, methylotrophic yeast strains are modified by transforming into these yeast strains with one or more, methylotrophic yeast strains to reduce glycosylation on proteins produced by the i.e., at least one, knock-in and/or knock-out vectors of the present invention as described herein above.

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methods include but are not limited to yeast capable of growth on methanol such as which are exemplary of this class of yeasts can be found in C. Anthony (1982), The yeasts of the genera Candida, Hansemula, Torulopsis, and Pichia. A list of species Biochemistry of Methylotrophs, 269. Pichia pastoris, Pichia methanolica, Pichia Methylotrophic yeast strains which can be modified using the present anomola, Hansemula polymorpha and Candida boidinii are examples of 23

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methylotrophic yeasts useful in the practice of the present invention. Preferred methylotrophic yeasts are of the genus *Pichia*. Especially preferred are *Pichia pastoris* strains GS115 (NRRL Y-1881); GS190 (NRRL Y-18014) disclosed in U.S. Patent No. 4,818,700; PPF1 (NRRL Y-18017) disclosed in U.S. Patent No. 4,812,405; PPY120H and yGC4; as well as strains derived therefrom.

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Methylotrophic yeast strains which can be modified using the present methods also include those methylotrophic yeast strains which have been genetically engineered to express one or more heterologous glycoproteins of interest. The glycosylation on the heterologous glycoproteins expressed from these previously engineered strains can be reduced by transforming such strains with one or more of the vectors of the present invention.

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The vectors of the present invention can be introduced into the cells of a methylotrophic yeast strain using known methods such as the spheroplast technique, described by Cregg et al. 1985, or the whole-cell lithium chloride yeast transformation system, Ito et al. Agric. Biol. Chem. 48:341, modified for use in Pichia as described in EP 312,934. Other published methods useful for transformation of the plasmids or linear vectors include U.S. Patent No. 4,929,555; Hinnen et al. Proc. Nat. Acad. Sci. USA 75:1929 (1978); Ito et al. J. Bacteriol. 153:163 (1983); U.S. Patent No. 4,879,231; Sreekrishna et al. Gene 59:115 (1987). Electroporation and PEG1000 whole cell transformation procedures may also be used. Cregg and Russel Methods in Molecular Biology: Pichia Protocols, Chapter 3, Humana Press, Totowa, N.J., pp. 27-30 (1998).

Transformed yeast cells can be selected by using appropriate techniques including but not limited to culturing auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype, or culturing in the presence of an antibiotic which is toxic to the yeast in the absence of a resistance gene contained in the transformants. Transformants can also be selected and/or verified by integration of the

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expression cassette into the genome, which can be assessed by e.g., Southern Blot or PCR analysis.

In one embodiment, a methylotrophic yeast strain is transformed with a vector which includes a nucleotide sequence coding for an α-1,2-manuosidase or a functional part thereof. The nucleotide sequence is canable of expressing the α-1,2-

functional part thereof. The nucleotide sequence is capable of expressing the α-1,2-mannosidase or the functional part in the methylotrophic yeast strain, and is, preferably, integrated into the genome of the methylotrophic yeast strain.

The expression of an α-1,2-mannosidase introduced in a methylotrophic yeast strain can be verified both at the mRNA level, e.g., by Northern Blot analysis, and at the protein level, e.g., by Western Blot analysis. The intracellular localization of the protein can be analyzed by using a variety of techniques, including subcellular fractionation and immunofluorescence experiments. An ER localization of an α-1,2-mannosidase can be determined by co-sedimentation of this enzyme with a known ER resident protein (e.g., Protein Disulfide Isomenase) in a subcellular fractionation estaining pattern characteristic of ER resident proteins, typically a perinuclear staining pattern.

To confirm that an α-1,2-mannosidase or a functional part thereof expressed in a methylotrophic yeast strain has the expected mannoso-trimming activity, both *in vitro* and *in vitro* says can be employed. Typically, an *in vitro* assay involves digestion of an *in vitro* synthesized substrate, e.g., Man<sub>8</sub>GlcNAc<sub>2</sub>, with the enzyme expressed and purified from a methylotrophic yeast strain, and assessing the ability of such enzyme to trim Man<sub>8</sub>GlcNAc<sub>2</sub>, to, e.g., Man<sub>8</sub>GlcNAc<sub>2</sub>. In *in vivo* assays, the α-1,2-mannosidase or a part thereof is co-expressed in a methylotrophic yeast with a glycoprotein known to be glycosylated with N-glycans bearing terminal α-1,2-linked mannose residues in such yeast. The enzymatic activity of such an α-1,2-linked mannose residues in the structures of the N-glycans of the number of α-1,2-linked mannose residues in the structures of the N-glycans of the

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glycoprotein. In both in vitro and in vivo assays, the composition of a carbohydrate group can be determined using techniques that are well known in the art and are illustrated in the Examples hereinbelow.

In another embodiment, a methylotrophic yeast strain is transformed with a vector which includes a nucleotide sequence coding for a glucosidase II or a functional part thereof. The nucleotide sequence is capable of expressing the glucosidase II or the functional part in the methylotrophic yeast strain, and is, preferably, integrated into the genome of the methylotrophic yeast strain.

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The enzymatic activity of a glucosidase II or a functional part thereof expressed in a transformed methylotrophic yeast strain can be assessed using a variety of assays. For example, methylotrophic yeast cells transformed with a sequence encoding a glucosidase II or a part thereof can be set to grow on solid medium containing a substrate of the glucosidase, e.g., 5-bromo-4-chloro-3-indolyl-a-D-glucopyranoside or 4-MU-a-D-Glc. When the enzyme is expressed by the *Pichia* and

15 secreted extracellularly, the substrate is acted upon by the enzyme, giving rise to detectable signals around the colonies such as blue color or fluorescent glow.
Alternatively, liquid culture medium containing the expressed protein molecules can be collected and incubated in test tubes with a substrate, e.g., p-nitrophenyl-α-D-glucopyranoside. The enzymatic activity can be determined by measuring the specific

product released. Moreover, *In vivo* assays can be employed, where a glucosidase II is co-expressed in yeast with a glycoprotein known to be N-glycosylated with glucose residues, e.g., influenza neuraminidase. The enzymatic activity of the glucosidase II can be measured based on the reduction of the glucose content in the sugar chain(s) of the glycoprotein.

In still another embodiment of the present invention, a methylotrophic yeast strain is transformed with an Ochl knock-out vector. As a result of the transformation and integration of the vector, the genomic Ochl gene in the yeast strains is disrupted.

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In a further embodiment of the present invention, a methylotrophic yeast strain is transformed with any combination of an  $\alpha$ -1,2-mannosidase expression vector, a glucosidase II expression vector, and an Oohl knock-out vector. Such modification can be achieved by serial, consecutive transformations, i.e., introducing one vector at a time, or alternatively by co-transformation, i.e., introducing the vectors simultaneously.

The modified methylotrophic yeast strains described herein above can be further modified if desired. For example, additional disruption of genes encoding any other *Pichia* mannosyltransferases can be made. Genes encoding mammalian enzymes can also be introduced to produce glycoproteins having hybrid- or complex-

type N-glycans, if desired.

Methylotrophic yeast strains which are modified by using the present methods, i.e., by transforming with one or more of the vectors of the present invention, form another embodiment of the present invention.

It should be understood that certain aspects of the present invention, especially the introduction of an intracellularly expressed or-1,2-mannosidase activity, are also useful to obtain a reduced glycosylation of the O-linked glycans on glycoproteins produced in a methylotrophic yeast, as it is known in the art that these O-linked glycans consist mainly of or-1,2-linked mannose residues. O-linked glycans as used herein refers to carbohydrate structures linked to serine or threonine residues

A further aspect of the invention is directed to methods of producing a glycoprotein with reduced glycosylation in a methylotrophic yeast, especially a glycoprotein heterologous to the methylotrophic yeast.

of glycoproteins.

"A glycoprotein" as used herein refers to a protein which, in methylotrophic yeasts, is either glycosylated on one or more asparagines residues or on one or more serine or threonine residues, or on both asparagines and serine or threonine residues.

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unmodified strain of the methylotrophic yeast.

In accordance with the present invention, the production of a glycoprotein methylotrophic yeast strain which has been previously modified in accordance with nucleotide sequence capable of expressing a glycoprotein can be introduced into a of interest with reduced glycosylation can be achieved in a number of ways. A

methylotrophic yeast strain can be transformed with one or more of the present knockpresent invention and capable of producing glycoproteins with reduced glycosylation. express a glycoprotein of interest, nor is the strain transformed with any of the vectors of the present invention, such yeast strain can be transformed, either consecutively or the present invention, i.e., a strain transformed with one or more of the vectors of the vectors of the present invention. Otherwise, if a methylotrophic yeast strain does not engineered to express a glycoprotein can be transformed with one or more of the in and/or knock-out vectors which also include a nucleotide sequence capable of Alternatively, a methylotrophic yeast strain which has already been genetically glycoprotein and one or more vectors of the present invention. Additionally, a simultaneously, with both a nucleotide sequence capable of expressing the 12 2 8

methylotrophic yeast can be made to include from 5' to 3', a promoter, a sequence The nucleotide sequence capable of expressing a glycoprotein in a encoding the glycoprotein, and a 3' termination sequence. Promoters and 3'

expressing a glycoprotein in the methylotrophic yeast strain.

termination sequences which are suitable for expression of a glycoprotein can include any of those promoters and 3' termination sequences described hereinabove. ង

additional sequences, e.g., signal sequences coding for transit peptides when secretion of a protein product is desired. Such sequences are widely known, readily available The nucleotide sequence for expression of a glycoprotein can include

and include Saccharomyces cerevisiae alpha mating factor prepro (cant), Pichia pastoris acid phosphatase (PHO1) signal sequence and the like. The nucleotide sequence for expression of a glycoprotein can be placed on a replicative vector or an integrative vector. The choice and construction of such

vectors are as described hereinabove.

glucosidase II expression unit. Alternatively, the nucleotide sequence containing the glycoprotein coding sequence is carried on a separate plasmid or integrated into the carried on the same replicative plasmid as a plasmid-borne  $\alpha$ -1,2-mannosidase or The nucleotide sequence capable of expressing a glycoprotein can be host genome.

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example, the cell culture medium is separated from the cells and the protein secreted Purification protocols can be determined by the nature of the specific protein to be purified. Such determination is within the ordinary level of skill in the art. For Glycoproteins produced can be purified by conventional methods.

from the cells can be isolated from the medium by routine isolation techniques such as precipitation, immunoadsorption, fractionation or a variety of chromatographic methods 15

invention include, e.g., Bacillus amyloliquefactens a-amylase, S. cerevisiae invertase,

Glycoproteins which can be produced by the methods of the present

numan angiostatin, human B7-1, B7-2 and B-7 receptor CTLA-4, human tissue factor, haemagglutinin, influenza neuraminidase, Bovine herpes virus type-1 glycoprotein D, plasminogen activator inhibitor-I, urokinase, human lysosomal proteins such as agrowth factors (e.g., platelet-derived growth factor), tissue plasminogen activator, Trypanosoma cruzi trans-sialidase, HIV envelope protein, influenza virus A ន

additional useful glycoproteins which can be expressed in the genetically engineered Pichia strains of the present invention, see Bretthauer and Castellino, Biotechnol. galactosidase, plasminogen, thrombin, factor XIII and immunoglobulins. For 23

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Appl. Biochem. 30: 193-200 (1999), and Kukuruzinska et al. Ann Rev. Biochem. 56: 915-44 (1987). Glycoproteins produced by using the methods of the present invention, i.e., glycoproteins with reduced glycosylation, are also part of the present invention.

Still another aspect of the present invention provides kits which contain one or more of the knock-in vectors, knock-out vectors, or knock-in-and-knock-out vectors of the present invention described above. More particularly, a kit of the present invention contains a vector capable of expressing a glucosidase II in a methylotrophic yeast, a vector capable of expressing the Ochl gene in a methylotrophic yeast, a vector capable of expressing both a glucosidase II and an α-mannosidase, a vector a vector capable of expressing the Ochl gene and capable of expressing either or both of a glucosidase II and an α-mannosidase, or any combinations thereof.

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The kit can also include a nucleotide sequence which encodes and is capable of expressing a heterologous glycoprotein of interest. Such nucleotide sequence can be provided in a separate vector or in the same vector which contains sequences for knocking-in or knocking out as described hereinabove.

In addition, the kit can include a plasmid vector in which a nucleotide sequence encoding a heterologous protein of interest can be subsequently inserted for transformation into and expression in a methylotrophic yeast. Alternatively, the knock-in or knock-out vectors in the kits have convenient cloning sites for insertion of a nucleotide sequence encoding a heterologous protein of interest.

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The kit can also include a methylotrophic yeast strain which can be subsequently transformed with any of the knock-in, knock-out or knock-in-and-knock-out vectors described hereinabove. The kit can also include a methylotrophic yeast strain which has been transformed with one or more of the knock-in or knock-out vectors. Furthermore, the kit can include a methylotrophic yeast strain which has been

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transformed with a nucleotide sequence encoding and capable of expressing a heterologous glycoprotein of interest.

The present invention is further illustrated by the following examples.

<u>Example 1</u> Introduction of a-1,2-Mannosidase to the ER-Golgl Border

#### 1.1 Plasmids

Plasmid	Promoter Enzyme	Enzyme	Tag
pGAPZMFManHDEL	GAP	T. reesei a-1,2-mannosidase	1
pGAPZMFManMycHDEL	GAP	T. reesei α-1,2-mannosidase   Myc	Myc
pPICZBMFManMycHDEL	AOXI	T. reesei α-1,2-mannosidase	Myc
pGAPZMFmManHDEL	GAP	mouse mannosidase IB	ı
		catalytic domain	
pGAPZMFmMycManHDEL	GAP	mouse mannosidase IB	Myc
	, and the second	catalytic domain	•

The Trichoderma reesei a-1,2-mannosidase gene has been isolated and described by Maras et al. (J. Biotechnol. 77,255-263, 2000). The sequence of this gene is available at NCBI Genbank under Accession No. AF212153. A construction fragment was generated by PCR using the pPIC9MFmanase plasmid (same as

- pPP1MFmds1 described by Maras et al. (2000)) as the template and using the following oligonucleotide primers: 5'-GACTGGTTCCAATTGACAAGC-3' (SEQ ID NO:2) and 5'-AGTCTAGATTACAACTCGTCGTGAGCAAGGTGGCCCCCG TCG-3' (SEQ ID NO:3). The resulting product contained the 3' end of the *Pichia pastoris* AOXI promoter, the prepro-signal sequence of the S. cerevisiae a-mating
- 15 factor, the open reading frame of the *Trichoderma reesei* α-1,2-mannosidase cloned in frame with the signal sequence, the coding sequence for HDEL, a stop codon and an *Xba* I restriction site. This fragment was digested with *Eco* RI and *Xba* I, removing the 5' sequences up to the mannosidase ORF, and then cloned into the vector pGAPZαA (Invitrogen, Baam, The Netherlands) which had been digested with *Eco* RI

and Xba I, thus restoring the fusion with the S. cerevistae a-mating factor signal

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sequence. The resulting plasmid was named pGAPZMFManHDEL and is graphically depicted in Figure 1. The ORF sequence of the MFManHDEL fusion in pGAPZMFManHDEL is set forth in SEQ ID NO: 14.

In order to introduce the coding sequence for a c-Myc tag between the

catalytic domain and the HDEL-signal, the 3' and of the ORP of T. reesei α-1,2mannosidase was PCR-amplified using a sense primer 5'CCATTGAGGACGCATGCCGCCC-3' (SEQ ID NO: 4) (containing an Sph I
restriction site) and an antisense primer

GTATCTAGATTACAACTCGTCGTGCAGATCCTCTTCTGAGATGAGTTTTTTGT

10 TCAGCAAGGTGGCCGCCCGTCGTGATGAAGAA (SEQ ID NO: 5) (containing the coding sequences of the c-Myc tag and the HDEL signal, followed by a stop codon and an Xba I restriction site). The resulting PCR product was digested with 5ph I and Xba I, purified by agarose gel electrophoresis and inserted into pGAPZMFManHDEL which had been cut with the same restriction enzymes, resulting in plasmid

15 pGAPZMFManMycHDEL. To put the ORF of pGAPZMFManMycHDEL under the control of the inducible AOXI promoter, the entire ORF was liberated from pGAPZMFManMycHDEL with Bst Bl and Xba I, and cloned in pPICZB (Invitrogen, Baarn, The Netherlands), resulting in pPICZBMFManMycHDEL.

Cloning of the mouse mannosidase IB catalytic domain with concomitant
addition of the coding sequence for a C-terminal HDEL-tag was done by PCR on a
mouse cDNA library (mRNA isolated from the L929 cell line induced with
cycloheximide and mouse Tumor Necrosis Factor. Average insert length of the cDNA
library was 2000 bp). The PCR oligonucleotide primers used were:
SAACTCGAGATGGACTCTTCAAAACACAAACGC3' (SEQ ID NO: 6) and

25 STTGCGGCCGTTACAACTCGTCGTGGACAGCAGGATTACCTGA3'
(SEQ ID NO: 7). The product contained a 5' Xho I site and the coding sequence for Cterminal HDEL-site, followed by a stop codon and a Not I site at the 3' end. The
product was cloned in pGAPZαA via the Xho I /Not I sites in the PCR product and the
vector, resulting in an in frame fusion of the mouse mannosidase catalytic domain

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with the S. cerevisiae a-mating factor signal sequence. The sequence of the entire open reading frame generated is set forth in SEQ ID NO: 15.

# 1.2 Yeast Transformation and Genomic Integration

Table 2

Parental strain	DNA transformed
GS115 (his4)	pGAPZMFManHDEL
	pPIC9MFManHDBL
	pPIC9mManHDEL
	pPIC9mMycManHDEL
	pGAPZmManHDEL
	pGAPZmMycManHDEL
GS115 (his4 complemented by	pGAPZMFManHDEL
pPIC9InfluenzaHA)	
	pGAPZmManHDEL
	pGAPZmMycManHDEL
PPY120H (his4 complemented by	pGAPZMFManMycHDEL
pPIC9sOCH1)	
	pPICZBMFManMycHDEL
yGC4 (his4 arg1 ade2 ura3	pPIC9InfluenzaNeuraminidase
complemented by	
pBLURA5'PpOCH1)	
	pGAPZMFManHDEL
	pPIC9Glucoseoxidase

All transformations to *Pichia pastoris* were performed with electroporation according to the directions of Invitrogen. Transformants of vectors carrying the Zeocin resistance gene were selected on YPD containing 100 µg/ml Zeocine

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(Invitrogen, Baarn, the Netherlands) and 1M sorbitol. Selection of transformants of pPIC9 derivatives was done on minimal medium lacking histidine and containing 1M sorbitol. Genomic integration of the expression cassettes was verified using PCR on genomic DNA purified from the Pichia strains using the Yeast Miniprep method

- 5 (Nucleon). In all cases concerning the *Trichoderma reeset* gene fusions, the primers used were the sense primer 5'-CCATTGAGGACGCATGCCGCGCGC3' (SEQ ID NO: 8), which annealed to the 3' half of the mannosidase ORE, and the antisense primer 3' AOXI 5'-GCAAATGGCATTCTGACATCCT-3' (SEQ ID NO: 9), which annealed to the AOXI transcription terminator that was present in all our expression constructs.

  For the control of genomic integration of the mouse mannosidase transgenes, PCR was
  - 10 For the control of genomic integration of the mouse mannosidase transgenes, PCR was done using the sense primer S'GAP S'GTCCCTATTTCAATCAATTGAA3' (SEQ ID NO: 10, annealing to the GAP promoter or S'AOXI S'GACTGGTTCCAATTGACAAGC3' (SEQ ID NO:11), annealing to AOXI promoter), and the antisense primer 3'AOXI (above). For the expression constructs
- 15 containing a Myc tagged Thehoderma reeset a.-1,2-mannosidase expression unit, further evidence for genomic integration was obtained using Southern Blotting with the entire MFManMycHDEL ORF (<sup>32</sup>P labelled using HighPrime, Boehringer Mannheim) as a probe.

# 20 1.3 Expression of α-1.2-mannosidase

Expression of an α-1,2-Mannosidase in GS115 strains expressing influenza virus haemagglutinin was verified by qualitative Northern blot. Expression of an α-1,2-Mannosidase in PPY120H strains was verified by anti-Myc Western blot.

Qualitative Northern Blot — Total RNA was purified from Pichla strains
and the yield was determined spectrophotometrically. Northern blotting was
performed according to standard procedures and an estimate of the quantity of RNA
loaded was made using methylene blue staining of the blot, visualizing the rRNA

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bands. The blot was probed with a Clal/Narl fragment of the mannosidase, labelled

SDS-PAGE and Western Blotting -- Total yeast cell lysates were prepared by washing the cells twice with PBS, followed by boiling in 1 volume of 2x

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with 32 Pusing HighPrime (Boehringer Mannheim).

Mannheim) were used at a concentration of 1µg/ml, and the rabbit anti-PDI antiserum standard procedures. The pellet was redissolved in 2x concentrated Laemmli loading (Stressgen) was used at a dilution of 1/500. The secondary antibodies were goat antiand followed by semidry electroblotting to nitrocellulose membranes. For Western buffer and the solutions were pH-corrected using Tris. SDS-PAGE was performed microcentrifuge prior to gel loading and only the supernatant was loaded. For the analysis of proteins secreted into the growth media, the proteins were precipitated rabbit IgG conjugated to peroxidase for the polyclonal (secondary antibodies from from 200 µl of these media using desoxycholate/trichloroacetic acid according to mouse IgG conjugated to alkaline phosphatase for the monoclonals and goat anticoncentrated Leernmli loading buffer for 5 min. The lysate was spun briefly in a Blotting, the 9E10 anti-Myc and the anti-HA mouse monoclonals (Boehringer Sigma). Detection was performed using the NBT/BCIP system for alkaline

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HDEL-tagged protein was retained intracellularly, both when expressed from the strong constitutive GAP promoter and when expressed from the strong inducible The results shown in Figure 4 indicated that the great majority of the AOXI promoter.

Imaging of the latter blot result was done on a Lumilager imaging device (Boehringer

Mannheim)

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phosphatase and the Renaissance substrate (NENBiosciences) for peroxidase.

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# 1.4 Localization of a-1,2-Mannosidase

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localization of the HDEL-tagged mannosidase, subcellular fractionation was carried Isopycnic sucrose density gradient centrifugation -- To determine the

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out using cells expressing the mannosidase-Myc-HDEL from the strong constitutive GAP promoter.

vortexing with 4.5 g glass beads in 1 ml lysis-buffer (50 mM Tris-HCL pH 7.5 Briefly, 0.5 g of wet weight yeast cells were lysed using 4 x 1 min

- differential centrifugation procedure. The P10000 pellet was solubilized in 0.5 ml of a of this washing step was added to the first supernatant. This lysate was subjected to a collected and the glass beads were washed once with lysis-buffer, and the supernatant containing 0.6 M sorbitol, 10 mM β-mercaptoethanol and 5 mM MgCl<sub>2</sub>). Between vortexing periods, the mixture was placed on ice for 5 min. The supernatant was
- gradient centrifugation was performed for 14 h at 180,000g in a Beckman SW 41 rotor Ultraclear ultracentrifuge tube (Beckman) of 14 x 89 mm. Subsequently, 1.5 ml each 60% sucrose solution in lysis buffer. This solution was placed at the bottom of an of sucrose solutions of 55, 50, 45, 42.5, 40, and 37.5% were carefully layered over each other. The tube was filled to the edge with 35% sucrose. Isopycnic sucrose 2 15
  - Western blots were treated with anti-HA, anti-Myc or anti-PDI ("PDI" for Protein fractions were collected from the top and partially dialysed from excess sucrose, Laemmli buffer, the samples were subjected to SDS-PAGE in triplicate and the in a Beckman Model L8-70 preparative ultracentrifuge. After completion, 1ml evaporated to dryness in a vacuum centrifuge. After redissolving the pellet in Disulfide Isomerase), respectively.

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(which is also targeted with a HDEL signal sequence) (Figure 5). In the same assay, MFManMycHDEL protein with the Protein Disulfide Isomerase marker protein the HA-tagged OCHI was distributed over the whole gradient, with the highest The results illustrated almost exact cosedimentation of the

the expected location (the ER-Golgi boundary) by the addition of an HDEL signal. In abundance in fractions having a density lower than that of the fractions containing the contrast, the mannosidase without HDEL, expressed from inducible alcohol oxidase I mannosidase and the PDI. This result indicated that the mannosidase was targeted to ಜ

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Immunofluorescence microscopy - To confirm the correct targeting of the mannosidase-Myc-HDEL, an immunofluorescence microscopy experiment was

5 performed.

Briefly, yeast cultures were grown to OD<sub>600</sub> in YPD (for pGAPZMFManMycHDEL) or in YMP following a YPGlycerol growth phase for pPICZBMFManMycHDEL. Formaldehyde was added to the yeast cultures to a final concentration of 4% and incubated for 10 min at room temperature. Cells were

- 10 pelleted and resuspended in 50mM potassium phosphate buffer pH 6.5 containing ImM MgCl<sub>2</sub> and 4% formaldehyde and incubated for 2h at room temperature. After pelleting, the cells were resuspended to an OD<sub>600</sub>=10 in 100 mM potassium phosphate buffer pH 7.5 containing 1mM MgCl<sub>2</sub> and EDTA-free Complete <sup>TM</sup> protease inhibitor cocktail (Boehringer Mannheim). To 100 μl of cell suspension, 0.6 μl of β-mercapto-than 120 μl of 20 μl of 20 motates added, followed by a
- ethanol and 20µl of 20,000 U/ml Zymolyase 100T (ICN) were added, followed by a 25 minute incubation with gentle shaking. The cells were washed twice in the incubation buffer and added to poly-lysine coated cover slips (these are prepared using adhesive rings normally in use for reinforcing perforations in paper). Excess liquid was blotted with a cotton swab and the cells were allowed to dry at 20°C. All
- blocking, antibody incubation and washing steps are performed in PBS containing
   0.05% bovine serum albumin. Primary antibodies are used at 2µg/µl and secondary antibodies conjugated to flurophores (Molecular probes) were used at 5µg/µl. The nucleus was stained with the nucleic acid stain HOECHST 33258. After fixation and cell wall permeabilization, the integrity of the yeast cell morphology was checked in phase contrast microscopy and after immunostaining, the slides were examined under a Zeiss Axiophot fluroresensce microscope equipped with a Kodak digital camera. Images were processed using Macprobe 4.0 software and prepared with Corel

The Golgi marker protein OCH1-HA gave the typical Golgi staining pattern described in the literature (speckle-like staining). Staining with the 9E10 monoclonal anti-Myc antibody, recognizing mannosidase-Myc-HDEL, gave a perinucleer staining pattern with some disparate staining in the cytoplasm, highly

5 indicative for an ER targeting (Figure 4).

Based on the foregoing experiments, it is concluded that the Trichoderma reesel mannosidase-Myc-HDEL was targeted to the ER-Golgi boundary.

#### Example 2

Co-expression of Mannosidase-HDEL

with Recombinant Glycoproteins

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Co-expression of Mannosidase-HDEL with the Trypanosoma cruzl trans-Sialidase

The cloning of a *Trypanosoma cruzi trans*-sialidase gene coding for an active *trans*-sialidase member without the C-terminal repeat domain has been 15 described by Laroy et al. (*Protein Expression and Purification* 20: 389, 2000) which is incorporated herein by reference. The sequence of this *Trypanosoma cruzi trans*-sialidase gene is available through NCBI Genbank under the Accession No. AJ276679. For expression in *P. pastoris*, the entire gene was cloned in pHILD2 (Invitrogen, San Diego, CA), creating pHILD2-TS. To allow better secretion, pPIC9-

- 20 TS was created in which *trans*-sialidase was linked to the prepro secretion signal of the yeast α-mating factor. Plasmids pPIC9-TSE and pCAGGS-prepro-TSE were created where the epitope E-tag was added to the C-terminal of the *trans*-sialidase to allow easy detection and purification. The construction of pHILD2-TS, pPIC9-TSE and pCAGGS-prepro-TSE has been described by Laroy et al. (2000), incorporated
- 25 herein by reference. The vectors used in the construction were made available through http://www.belspo.be/bccm/lmbp.htm#main for pCAGGS (No. LMBP 2453), Invitrogen, San Diego, CA for pHILD2 and pPIC9, and Pharmacia Biotech for pCANTAB-5E.

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Plasmid pPIC9-TSE was linearized with Sstl and was transformed into P. plasmid pGAPZMFManHDEL, establishing a strain co-expressing Mannosidaseinstructions (Invitrogen). One of the transformants was further transformed with pastoris GS115 (his4) strain by electroporation according to the manufacturer's

Fermentation and protein purification was according to the procedures HDEL and the Trypanosoma cruzi trans-sialidase.

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described by Laroy et al. (2000).

Callewaert et al., Glycobiology 11, 4, 275-281, 2001. Briefly, the glycoproteins were glycans were analysed by electrophoresis on a 36 cm sequencing gel on an ABI 377A bound to the PVDF membrane in the wells of a 96-well plate, reduced, alkylated and submitted to peptide-N-glycosidase F deglycosylation. The glycans were derivatised with 8-amino-1,3,6-pyrenetrisulfonic acid by reductive amination. Subsequently, the Purified trans-sialidase was subject to carbohydrate analysis according to excess free label was removed using Sephadex G10-packed spin columns and the 2

derived from 1 µg of the purifed recombinant glycoproteins were used as the substrate. IU of the  $\alpha$ -1,2-mannosidase is defined as the amount of enzyme that releases 1 µmol 255-63, 2000) were also performed in 20 mM sodium acetate pH=5.0. The glycans DNA-sequencer and detected using the built-in argon laser. Digests with 3 mU/ml purified T. reeses a-1,2-mannosidase (described by Maras et al., J. Biotechnol. 77, 2

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As can be seen in Figure 6, panel B, the major N-glycan on trans-sialidase was MangGlcNAc2 (Compare with panel P, representing an analysis of the N-glycans peak is Man<sub>5</sub>GlcNAc<sub>2</sub>). In vitro, this glycan was digestible to Man<sub>5</sub>GlcNAc<sub>2</sub> with a-1,2-mannosidase (Figure 6, panel C). In the N-glycan profile of the trans-sialidase of bovine RNAseB. The one but last peak in this profile is MangGlcNAcs, the first of mannose from baker's yeast mannan per minute at 37°C and pH=5.0. co-expressed with mannosidase-HDEL, the major peak corresponded to MansGlcNAcs (Figure 6, panel D).

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Co-expression of Mannosidase-HDEL with the Influenza A virus baemagglutinin

The Influenza A virus haemagglutinin was known to be glycosylated in (Saelens et al. Eur. J. Biochem. 260: 166-175, 1999). The effect of a co-expressed Pichia pastoris with high-mannose N-glycans containing 9-12 mannose residues

- mannosidase on the N-glycans of the baemagglutinin was assessed in an N-glycan Trichoderma enzyme (having a temperature optimum of 60°C) with a mammalian mouse mannosidase IB from a mouse cDNA-library was cloned and tagged with a HDEL signal by PCR amplification. This ORP was cloned after the prepro-signal mannosidase having a temperature optimum of 37°C, the catalytic domain of the profiling method described below. In addition, to compare the efficiency of the Ś
- sequence of the S. cerevisiae  $\alpha$ -mating factor under the control of the GAP promoter. Expression of the mannosidase-HDEL transgenes on the mRNA level was confirmed by qualitative Northern blotting. 2
- haemagglutin was subjected to PNGase F digestion as described by Saelens et al. Eur. mannosidase-HDEL or the mouse mannosidase IB-HDEL according to the procedure I. Biochem. 260: 166-175, 1999. The proteins and glycans were precipitated with 3 The haemagglutinin was expressed and purified from a non-mannosidase expressing control strain and from a strains co-expressing the Trichoderma reesei described by Kulakosky et al. Glycobiology 8: 741-745 (1998). The purified 2
- volumes of ice-cold acetone and the glycans were extracted from the pellet with 60% 1,3,6 pyrenetrisulfonic acid by adding 1 µl of a 1:1 mixture of 20 mM APTS in 1.2M citric acid and 1M NaCNBHs in DMSO and incubating for 16h at 37°C at the bottom of a 250 µl PCR-tube. The reaction was stopped by the addition of 10 µl deionized methanol. Following vacuum evaporation, the glycans were labeled with 8-amino-2
- a microspin-column by centrifugation in a swinging bucket rotor, which provided for a water and the mixture was loaded on a 1.2 cm Sephadex G10 bed packed to dryness in abletop centrifuge. This elution process was repeated twice and all the eluates were flat resin surface. After loading, 50 µl deionised water was carefully added to the resin bed and the spin column was briefly centrifuged for 5 seconds at 750g in a 23

Bioscience), serving as an internal reference standard. This mixture was loaded on a pooled and evaporated to dryness in a Speedvac vacuum centrifuge (Savant). The labeled glycans were reconstituted in 1.5 µl gel loading buffer containing 50% formamide and 0.5 µl Genescan 500<sup>™</sup>, labeled with rhodamine (Perkin Elmer

- catalyzed by the addition of 200 µl 10% ammononiumpersulfate solution in water and 20 μl TEMED. The gel was of the standard 36 cm well-to-read length and was run on DNA-sequencing gel containing 10% of a 19:1 mixture of acrylamide:bisacrylamide (Biorad, Hercules, CA, USA) and made up in the standard DNA-sequencing buffer (89 mM Tris, 89 mM borate, 2.2 mM EDTA). Polymerization of the gel was S
  - 8h at 1250 V without heating. This methodology gives a limit of detection of 10 fmol gel was done at 1000 V for 15 min, and after loading, the gel was electrophoresed for an Applied Biosystems Model 373A DNA-sequencing apparatus. Prerunning of the per peak. The data were analysed with Genescan 3.0 software. 2

As shown in Figure 7, the Trichoderma reese  $\alpha$ -1,2-mannosidase

provided the most complete reduction in the number of  $\alpha$ -1,2-mannoses present on the N-glycans.. The N-glycan processing by mouse mannosidase IB-HDEL was less efficient than by the Trichoderma reesei a-1,2-mannosidase. 15

Despite the efficient removal of a-1,2-mannoses from the N-glycans of haemagglutinin, no Man, GlcNAc, was obtained. Even after digestion of the Nglycans with 3 mU of purified Trichoderma reesei a-1,2-mannosidase, only

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initiating OCH1 α-1,6-mannosyltransferase enzymatic activities. OCH1 was observed to be localized to very early part of the Golgi apparatus and could act on the N-glycans Man<sub>6</sub>GlcNAc<sub>2</sub> was obtained as the smallest sugar chain. These results indicated that the remaining residues were possibly  $\alpha$ -1,6-linked mannoses, originating from the

efficiently modified by the a-1,6-mannosyltransferase, an inactivation of the OCH1 Man<sub>5</sub>GlcNAc<sub>2</sub> by the mannosidases-HDEL. Thus, for proteins whose glycans are of haemagglutinin before complete digestion of the MangGlcNAc2 precursor to 23

gene coding for the transferase would be desirable in order to obtain proteins with MansGlcNAc2.

#### Example 3

## Inactivation of the Pichla Ochl Gene:

incorporated herein by reference. This sequence shows all typical features of an a-A Pichia pastoris sequence was found in the GenBank under Accession 1,6-mannosyltransferase and is most homologous to the S. cerevisiae OCHI, thus No. B12456 and was described in Japanese Patent Application No. 07145005, referred to herein as the Pichia pastoris Ochl gene.

ended with T4 polymerase, then cut with Xbal, releasing a fragment containing the 5' http://www.kgi.edu/html/noncore/faculty/cregg/cregg.htm), which had been cut with pUC18 to obtain plasmid pUC18pOch1. pUC18pOch1 was cut with HindIII, blunt-First, the full ORF of the Pichia pastoris Ochl gene was PCR cloned in part of the Pichia pastoris Ochl gene. This fragment was ligated into the vector Eco RI, blunt-ended with T4 polymerase, and then cut with Nhe I. This ligation pBLURA IX (available from the Keck Graduate Institute, Dr. James Cregg,

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Figure 9. As a result of the single homologous recombination, the Ochl gene on the Disruption of this Pichia OCH1 gane in the Pichia genome was achieved by single homologous recombination using pBLURA5'PpOCH1, as illustrated in

generated pBLURA5'PpPCH1, as shown in Figure 8.

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- the first one third of the full Och1 ORF, the other had a full Och1 ORF without a Och1 Pichia chromosome was replaced with two Och1 sequences: one consisted only about promoter. Single homologous recombination was achieved as follows. Cells of the Pichia strain yGC4 were transformed by electroporation with pBLURAS'PpOCH1 which had been linearized with the single cutter Bst Bl. About 500 transformants ຊ
- and histidine and incubation at 27°C. Thirty-two of these transformants were picked and re-selected under the same conditions. Twelve clones were further analyzed for were obtained on minimal medium containing 1M sorbitol, biotin, arginine, adenine correct genomic integration of the cassette by PCR. Seven of the twelve URA prototrophic clones contained the cassette in the correct locus. 22

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clone and three supertransformants also expressing the ManHDEL were evaluated in pGAPZMFManHDEL to produce "supertransformants". Both the Ochl-inactivated cell wall glycan analysis as follows. Yeast cells were grown in 10 ml YPD to an One of the Och1-inactivated clones was also further transformed with

- analysis using DSA-FACE as described by Callewaert et al. (2001) Glycobtology 11, sodium citrate buffer pH7 for 90 min at 120 °C and recovery of the supernatant after OD600=2 and mannoproteins were prepared by autoclaving the yeast cells in 20 mM cold methanol. The protein preparation obtained in this way was used for N-glycan centrifugation. Proteins were precipitated from this supernatant with 3 volumes of Ś
- expressed the HDEL-tagged a-1,2 mannosidase, the production of MansGlcNAc2 was of a reduced activity of the Ochl enzyme. In all three supertransformants which also glycan in the Ochl-inactivanted clone as compared to parent strain yGC4, indicative 275-281. As shown in Figure 10, there was an increased amount of ManaGlcNAc2 observed. Furthermore, upon digestion of the same glycan mixtures with 3 mU/ml 2
- purified recombinant Trichodarma reeset a-1,2-mannosidase, more ManyGlcNAc2 was formed in the strain transformed with pBLURAS'PpOCH1 than in the parent strain (Figure 11, compare panel 2 and 3). 2

indicate that the production of glycoproteins with Man, glycans could be facilitated by recombinantly produced proteins such as haemagglutinin from cells expressing a-1,2-These results confirmed that the lack of a production of Mans glycans on mannosidase were due to the activity of the Och1 protein. These results further the inactivation of the Och1 gene

### Example 4 Expression of Glucosidase II in *Pichia pastoris*

# 4.1. Amplification of the GLSII alpha subunit ORP from S. cerevisiae.

- Genomic DNA was prepared from the S. cerevisiae strain INVS (α, leu2-3, 112 his3 a1, trpl-289, ura3-52), using the Nucleon kit (Amersham). A touch-down PCR reaction was performed using this genomic DNA as template and the LA TaKaRa polymerase (ImTec Diagnostics). The sequence of the PCR primers was based on the known sequence of the S. cerevisiae GLSII ORF:
- 10 Sense primer: 5' CCG CTC GAG ATG GTC CTT TTG AAA TGG CTC 3'
  (SEQ ID NO:12)

Antisense primer: 5' CCG GGC CCA AAA ATA ACT TCC CAA TCT TCA

15 G 3' (SEQ ID NO:13)

# 4.2 Cloning of the S. cerevisine educosidase II ORF into Pichia pastoris expression

Construction of the glucosidase II expression vectors — The PCR fragment

20 was digested with Xho UApa I and ligated into the pGAPZA vector (Invitrogen),
thereby placing the ORF under the transcriptional control of the GAP promoter.

Using this strategy, the myc and the His6 tag were placed in frame to the C-terminus

of Glucosidase II, creating pGAPZAGLSII. The complete ORF of pGAPZAGLSII

was then sequenced to ensure that no mutations were generated in the PCR reaction.

The sequence of the vector pGAPZAGLSII was set forth in SEQ ID NO: 18. The GLSII ORF from the pGAPZAGLSII vector was cloned into vector pPICZA (Invitrogen) to create pPICZAGLSII, thereby placing the ORF under the transcriptional control of the AOXI promoter. The GLSII ORF from the pGAPZAGLSII vector was cloned into vector pAOXZZA, thereby placing the ORF

under the transcriptional control of the AOX2 promoter. This vector was created by

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replacing the multi cloning site of vector pAOX2ZB with the multi cloning site of pPICZA. Vector pAOX2ZB was generated by replacing the AOX1 promotor of pPICZB by the AOX2 promotor region of the AOX2 gene (Martinet et al., Biotechnology Letters 21). The AOX2 promotor region was generated by PCR on

- biotechnology Leachs 21). The AUXZ promotor region was generated by PCK on Pichla genomic DNA with the sense primer S'GACGAGATCTTTTTTCAGACCATATGACCGG 3' (SEQ ID NO: 26) and the antisense primer S'GCGGAATTCTTTTCTCAGTTGATTTGTTTGT 3' (SEQ ID NO: 27). The GLSII ORF from the pGAPZGLSII vector was cloned into vector pYPT1ZA to create pYPTIZAGLSII, thereby placing the ORP under the
- transcriptional control of the YPT1 promoter. Vector pYPTZA was created by replacing the AOX1 promoter of pPICZA by the YPT1 promoter present on the plasmid pIB3 (GenBank accession number AF027960)(Sears et al., Yeast 14, pg 783-790, 1998). All constructs contain the phleomycin resistance gene. The resulting final expression vectors (pGAPZAGLSII, pAOX2ZAGLSII, pPICZAGLSII and
  - pyPT1ZAGLSII) are depicted in Figures 12-15.

    Similar expression vectors were constructed, carrying the Ampicillin resistance marker and the Pichia ADE1 selection marker. In principle, the Zoocin resistance expression cassette of the plasmids pAOX2ZAGLSII, pGAPZAGLSII and pYPT1ZAGLSII was replaced by the Ampicillin and Pichia ADE1 cassette of the
- vector pBLADE IX (Cregg, J.M.) to result in the vectors pAOX2ADEIgIsII, pGAPADEIgIsII and pYPT1ADEIgIsII. Vector pPICADEIgIsII was obtained by inserting the glucosidase II open reading frame into the multiple cloning site of the vector pBLADE IX (Cregg, J.M.). The resulting final expression vectors (pGAPADEIgIsII, pAOX2ADEIgIsII, pPICADEIgIsII and pYPT1ADEIgIsII) are
- Adding the ER retention tag HDEL to Glucosidase II expression vectors—
  The following primers were used to generate an HDEL-containing PCR fragment:
  Primer 1: 5'GCG GGT CGA C/CA C/GA A/CT G/TG A/GT TTT AGC CTT
  Sal 1 H D E L stop

depicted in Figures 16-20.

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AGA CAT GAC 3' (SEQ ID NO.28)

Primer 2: 5'CAG GAG CAAA GCT CGT ACG AG 3' (SEQ ID NO.29)

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fragment of 225 bp was cut with Sal I/Spl I and ligated into the Sal I/Spl I opened pGAPZAGLSII with Taq pol., at 60°C. The PCR fragment of 225 bp was cut with Sal I/Spl I and ligated into the Sal I/Spl I opened pGAPZAGLSII vector, creating plasmid pGAPZAgIsIIHDEL. The sequence of plasmid pGAPZAgIsIIHDEL is set forth in SEQ ID NO: 24. The construction strategy and the resulting final expression vectors (pGAPZAgIsIIHDEL and pGAPADEIgIsIIHDEL) are depicted in Figures 20-21.

# 4.3 Transformation of a Pichla pastoris strain,

Transformation was performed using the conventional electroporation techniques, as described by Invitrogen. Cells of the *Pichia pastoris* strain PPY12-OH were transformed with pGAPZGLSII which had been cut with the single cutter *Avr* II. Transformants were selected based on their resistance to zoocin.

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Genomic analysis of the transformants — Genomic DNA was prepared from some zeocin resistant Pichia transformants. A PCR reaction was performed on the genomic DNA in order to determine whether or not the glucosidase II gene was integrated into the yeast genome. PCR was performed using Taq DNA polymerase (Boehinger) (2.5 mM MgCl<sub>2</sub>, 55°C for annealing). The primers were the same as the ones we used for the amplification of the ORF on S. cerevisiae genomic DNA. pGAPZAGLSII transformants were confirmed by the presence of a specific PCR product indicative of the glucosidase II ORP.

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# 4.4 Expression and secretion of the S. cerevisiae glucosidase II alpha subunit in Pichia pastoris

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Analysis at the transcriptional level — RNA was prepared from the transformants which scored positive after the genomic analysis. RNA was prepared using acid phenol. From each sample, 15 µg of RNA was loaded on a formaldehyde

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agarose gel. After electrophoresis the RNA was blotted on a Hybond N membrane. The membrane was hybridizing using a radioactive probe, which consists of a 344 bp glucosidase II specific fragment, corresponding to the 3' region of the glucosidase II ORF. No signals were detected with non-transformed control strains, whereas clear

5 signals were observed with transformants.

Analysis at the protein level using a double membrane assay – A nitrocellulose membrane was placed on a buffered dextrose medium (BMDY). On top of that nitrocellulose membrane, a cellulose acetate membrane was placed. Pichia transformants of pGAPZAGLSII were streaked on the cellulose acetate and grown for

10 a few days. The yeast cells remained on the cellulose acetate, while the secreted proteins crossed this membrane. As such the secreted protein was captured onto the nitrocellulose membrane. After a few days the cellulose acetate, containing the yeast colonies, was removed. The nitrocellulose membrane was analyzed for the presence of glucosidase II using anti-myc antibody. Most of the transformants gave a clear signal as compared to a faint, hardly visible signal with the WT, non-transformed strain.

Extracellular expression – PPY12-OH transformants of the construct pGAPZAGLSII(mychis6) (strains 12, 14 and 18) and transformants of the construct pGAPZAGLSII(mychis6) (strains H1, H2 and H3) were grown for 2 days on 2x10 ml BMDY medium. These 6 transformants earlier scored positive both on the genomic level (PCR on gDNA) and on the RNA level (Northern blot). The culture medium was collected by centrifugation and concentrated with Vivaspin columns to about 1 ml. Proteins from this concentrate were precipitated with TCA, resuspended in Laernmil buffer and loaded for SDS-PAGB analysis. Proteins were blotted to nitrocellulose membrane. The blot was incubated overnight with anti-myc Ab. The

nitrocellulose membrane. The blot was incubated overnight with anti-myc Ab. The secondary Ab was linked to peroxidase. Using the Renaissance luminiscence detection kit (NEN) and a light sensitive film (Kodak), a strong band at about 110 kDa was observed for the transformants 12, 14 and 18, indicating that GLSII was expressed and secreted from these transformants. No signal was obtained for the transformants

H1-3, which indicate that the HDEL tag, which was added C-terminally to the GLSII ORF, resulted in an ER localization of the protein, preventing GLSII to be secreted into the growth medium.

washed, resuspended into a minimal volume (50 mM Tris.HCl pH 7.5, 5% glycerol) Intracellular expression - The 6 transformants and the WT strain were grown for 2 days in 500 ml BMDY. The cells were collected by centrifugation, centrifugation steps (low speed centrifugation (2000-3000g)). Membranes were obtained from the supernatant through ultracentrifugation. The pellets were and broken using glass beads. The cell debris was removed through several S

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luminescence detection, a band at about 110 kDA was observed with the GLSIIHDEL checked using anti-myc Ab and peroxidase conjugated secondary Ab. Following the were blotted on a nitrocellulose membrane. The intracellular GLSII expression was resuspended in Laemmil buffer and loaded for SDS-PAGE analysis. The proteins recombinant GLSII when expressed with a C-terminal HDEL tag. No GLSII was expression strains. These results clearly indicate the intracellular presence of the transformants (H1 and H3, faint signal for H2), but not with the WT and GLSII detected intracellularly when this tag was not present.

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# 4.5 Purification and activity assays of the recombinant glucosidase II alpha

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A GLSII assay was set up as follows and was tested using a commercially available yeast alpha-glucosidase (Sigma) as a positive control. Composition: 70 µl 80 mM phosphate-citrate buffer pH 6.8, 7 µl 250 mM glucopyranoside (1 µM). Three assays were performed: one with 1 unit commercial enzyme, one without the enzyme and one with the enzyme but without the substrate. mannose, 3.5 µl 250 mM 2-deoxy-D-glucose, 0.8 µl 4-MeUmbelliferyl-alpha-D-

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The assay mixture was incubated overnight at 30°C. When illuminated with UV, only

the reaction mixture with both the enzyme and the substrate showed fluorescence

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(Figure 22). This indicates that the assay was very specific in detecting the activity of the alpha-glucosidase.

WT PPY12-OH, strain 18 and strain H3 were grown during 2 days in 2x10

ml growth medium. Cells were spun down and medium was adjusted to 300 mM

performed according to the manufactures recommendations. Protein was eluted from Medium was loaded onto a Ni-NTA spin column (Qiagen) and the purification was NaCl and 10 mM imidazol and concentrated with Vivaspin columns to 0.5-1ml. Ś

imidazol pH 8.0). From each eluate, 20 µl was assayed for its glucosidase II activity. the column in 2x100 µl elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM

0.33 units of the commercial enzyme diluted in 20 µl of the elution buffer was used as 2

a positive control. Pluorescence was observed with the positive control and the clute results indicate that the recombinant S. cerevisiae GLSII alpha subunit, secreted by of strain 18, the strain which secreted the enzyme into the growth medium. These

Pichia pastoris, was a functionally active enzyme. The activity was not seen in the

WT (untransformed) strain, nor in strain H3 as the GLSII was expressed intracellularly (Figure 23). These results also indicate that the beta subunit is not necessary for the 2

functionality of the alpha part of the protein.

#### both Glucosidase II and Mannosidase Creating Pichia Strains Expressing

Transformants were selected on YPDSzeo.

Strain GS115 was transformed with pGAPZGLSII and pGAPZglsIIHDEL.

Strain yGC4 was transformed with the following constructs, respectively:

(1) pGAPADEgIsII and pGAPADEgIsIIHDEL, selection on synthetic

sorbitol medium without adenine;

(2) pGAPZMFManHDEL: selection on YPDSzeo; and

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(3) pGAPZMFManHDEL/pGAPADEgIsIIHDEL: selection on synthetic

sorbitol medium without adenine and with zeocin.

Strain yGC4 with OCH1 knock-in and expressing MFmannosidaseHDEL

was transformed with pGAPADEgIsII and pGAPADEgIsIIHDEL. Selection of

transformants was done on synthetic sorbitol medium without adenine and uracil. 2

expression vector(s) integrated into the genome, determined by PCR, were obtained. For all transformations, colonies were obtained. Transformants with the

Expression of GLSII from some of these transformants was observed.

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SEQUENCE LISTING

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SEQ ID NO: 1 HDEL (peptide)

SEQ ID NO: 2 5'-GACTGGTTCCAATTGACAAGC-3'

SEQ ID NO: 3

5-AGTCTAGATTACAACTCGTCGTGAGCAAGGTGGCCGCCCCG TCG-3'

**SEQ ID NO: 4** 

2

CCATTGAGGACGCATGCCGCGCCC

SEQ ID NO: 5 GTATCTAGATTACAACTCGTCGTGCAGATCCTCTTCTGAGATGAGTTTTTGT TCAGCAAGGTGGCCGCCCGTCGTGATGATGAA

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SEQ ID NO: 6
AACTCGAGATGGACTCTTCAAAACACAAACGC

SEQ ID NO: 7 TTGCGGCCGCTTACAACTCGTCGTGTCGGACAGCAGGATTACCTGA ន

SEQ ID NO: 8 CCATTGAGGACGCATGCCGCGCC

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SEQ ID NO: 9
GCAAATGGCATTCTGACATCCT

SEQ ID NO: 10 GTCCCTATTTCAATCAATTGAA

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SEQ ID NO:11 GACTGGTTCCAATTGACAAGC

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SEQ ID NO:12 CCG CTC GAG ATG GTC CTT TTG AAA TGG CTC

SEQ ID NO:13 CCG GGC CCA AAA ATA ACT TCC CAA TCT TCAG

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#### SEQ ID NO: 14

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The ORF sequence of the MFManHDEL fusion in pGAPZMFManHDEL:

gctggggctcgtcggcaatcgatggcttggacacggctatcctcatgggggatgccgacattgtgaacacgatocttcagta gcaggctcasacactggccaacggcctcaaggttgcgttcaccactcccagcggtgtcccggaccctaccgtcttcttcaac gacctgacgggaaacccgcagtatgcccagcatgcagaagggcgagtcgtatctccgaaagggaagcccgg canangtacattgactttggnatcangctgccagctcglactttggcacglacacccagnoggcutctggnateggccccga cgacteceaagtggeaggaacctggegtggaagegttgeegttgeegttgeaggaegeatgeogegeegeagegegtaag algagatticcticasittitactgctgttitaticgcagcatcctccgcattagctgctccagtcsacactacaacagaagatgaa gcacaaataacgggttattgtttataaatactactattgccagcattgctgctaaaggaggagggtatctctcgagaaaagag gtogiggaacgettaccacaittigeetticeccaigaogactecacoggicageaacagettigaigaigagagaaacg octactiglooggagaagiggtgoatclagcaacaacglogcigaaatiggaagootggtgclogagtggacaoggttgago cteatggacagettetacgagtacotgateaagatgtacotgtacgacceggttgegtttgeacactacaaggategotgggt gacagictacgicgaaacicaggacattiggccagittiggrggiggcaacticatctigggaggcattcicgaacgag acggracaaattocggrtgaagctgtcatcggtactcagattagaaggggatttcgatgttgctgttttgccattttccaaca ggcotgctttctgcctatgacctgttgcgaggtcctttcagctccttggcgacaaaccagaccctggtaaacagccttctgag aggeatggeetggeetggtaggaacgtttgtcageacgageaacggtacetttcaggatageagggggggggtggteegge ccttggtgccgactcgaccattgggcatctcggctctcaccgtcgacgcaaggacttgaccttttgtcttcgtacaacg aggettegegtgggacagegtgaeggggegectggetggttegeegeetegteccagtecgggttetaetegtegea tecateaacgacgfgacgcaaggecaacggggggggggggcetetgaegatafggaagagettetggtttgecgateaa gtafgcgracctgatcttfgcggaggcggatgtgcaggfgcaggccaccggrgggaacaaatttgtctttaacacgga ggegeacccctttageatocgtteateateaegaegggggggggeggeteacdgsteaegaegagtg*taa* 

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#### SEQ ID NO: 15

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The ORF sequence of the MFmManHDEL fusion in pGAPZMFmManHDEL:

30 argagatticciteaattitacigetgittiaticgeageatecteegeattagetgeteeagteaacactacaacagagaatgaa aeggeacaaaatteeggetgaagetgtacteagattiagaaggggatticgagtgetgetgetgetgetticcaaca 5.4

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agtggggctgggaagcagcactggctattgagaagtcgtgccggggtagcgggggtttictggggttactggggtatcaggggtatacg
ccocgaccoctgtgcatgacgacggcagcagagcttttcttgctgaaacattaaaaaacttgaacagctgttctggggg
tgaccttctacctttagaccactgggggtttaacaacagaggcgcaccctctgccggtgttgcgcttagccaacagcactttic
aggtaatcctgctgccgacacgacgagttgaa

# 25 SEQ ID NO: 16 pAOX2ZAGLSII

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acgatectttagagaatgtgetteatgetaccataattaaaactataccaagattggagggegatgatatageegtteagtteee attetetetetetettiittaeaggateaeteagiaaggiteaetataaatgagaaagagagaatgeeaaeeaaeageagettig tentetettenen augsteen augagneet gang pange onte pacangan atte angang ng og na cagta trceaceafteceacttcctteagceaeaasceaectgtgeactcaftctggtcgeaeaeaettctafftttgtcactttcaaectccac tacacattgcaaacctcttccttctatttcggatcaactgtattgactacattgatctttttaacgaagtttacgacttactaaatcc antggctcgtntgccnattggtcttcttfaccgctttttcgcatgcgttfaccgactatchattanagnagtgtgcgcantctgggt tttgccatagaaacagggtttaigcagaaaatattgccaaaictcatcactgctattacaaagtggacgccgagtctattgcac tgcagacacatttcatcttcgaaacggtgatgtatccgtagaaatctttgctgaacctttcaattgaaagtttactggcaaaatg aanagnagcaaccaaaatcccttattgtccttttctgatcagcatcaaagaatattgtcttaaaacgggctttaactacattgttc ccacaaaacaaatcaactgagaaaagaattcacgtggcocagcoggoogtotoggatoggtacotogagatggtecttttga S 2

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# 20 SEQ ID NO: 17 pAOX2ADE1glsII

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cggcatagtatatcggcatagtataatacgacaaggtgaggaactaaac

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GCGGAATTCTTTTCTCAGTTGATTTTGTTTGT

SEQ ID NO: 28

GCG GGT CGA CCA CGA CGA ACT GTG AGT TTT AGC CTT AGA CAT GAC 13

SEQ ID NO: 29 CAG GAG CAAA GCT CGT ACG AG

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We claim:

functional part thereof in a methylotrophic yeast strain, comprising a nucleotide 1. A vector capable of expressing an a-1,2-mannosidase or a

sequence coding for said  $\alpha$ -1,2-mannosidase or said functional part.

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2. The vector of claim 1, wherein said  $\alpha$ -1,2-mannosidase is a protein

from a fungal species.

3. The vector of claim 2, wherein said fungus is Trichoderma reesel.

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4. The vector of claim 1, wherein said  $\alpha$ -1,2-mannosidase is a protein from a mammalian species.

2

5. The vector of claim 4, wherein said  $\alpha$ -1,2-mannosidase is murine  $\alpha$ -1,2-mannosidase IA or IB.

6. The vector of claim 1, wherein said a-1,2-mannosidase or said

functional part is tagged with an ER-retention signal.

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7. The vector of claim 6, wherein said ER-retention signal comprises peptide HDBL.

said a-1,2-mannosidase or said functional part is operably linked to a promoter and a 22

8. The vector of claim 1, wherein the nucleotide sequence coding for

3' termination sequence.

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The vector of claim 8, wherein said promoter is the promoter of a gene selected from the group consisting of AOXI, AOXII, GAP, and FLD.

- 10. A vector selected from the group consisting of
- 5 pGAPZMFManHDEL, pGAPZMFManMycHDEL, pPICZBMFManMycHDEL, pGAPZmManHDEL, pGAPZmMycManHDEL, pPIC9mMycManHDEL and pGAPZmMycManHDEL.
- 11. A vector capable of expressing a glucosidase II or a functional part thereof in a methylotrophic yeast strain, comprising a nucleotide sequence coding for said glucosidase II or said functional part.

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12. The vector of claim 11, wherein said glucosidase II is a protein from a fungal species.

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- The vector of claim 12, wherein said fungus is Saccharomyces cerevistae.
- 14. The vector of claim 11, wherein said glucosidase II is a protein
- 20 from a mammalian species.
- The vector of claim 11, wherein said glucosidase II or said functional part is tagged with an ER-retention signal.
- 25 16. The vector of claim 15, wherein said ER-retention signal comprises peptide HDEL.

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17. The vector of claim 11, wherein the nucleotide sequence coding for said α-1,2-mannosidase or said functional part is operably linked to a promoter and a 3' termination sequence.

5 18. The vector of claim 17, wherein said promoter is the promoter of a gene selected from the group consisting of AOXI, AOXII, GAP, and FLD.

 A vector having the designation pGAPZAGLSII, pPICZAGLSII, pAOXZZAGLSII, pYPTIZAGLSII, pGAPADEgIsII, pPICADEgisII, 10 pAOX2ADEgistI, pYPTIADEgistI, pGAPZAgistIHDEL and pGAPADEgistIHDEL.

20. A vector for disrupting the Och1 gene in a methylotrophic yeast strain, comprising a portion of the Och1 gene and a selectable marker gene, wherein said portion of the Och1 gene and said selectable marker gene are linked in such a way to effect the disruption of the genomic Och1 gene in said methylotrophic yeast strain.

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A vector having the designation pBLURA5'PpOCH1.

22. A method of reducing the glycosylation on proteins produced from 20 a methylotrophic yeast, comprising transforming said yeast with any one of the vectors of claims 1-21.

23. The method of claim 22, wherein said yeast is Pichia pastoris.

25 24. The method of claim 23, wherein said yeast is a Pichia pastoris strain selected from GS115 (NRRL Y-15851), GS190 (NRRL Y-18014), PPF1 (NRRL Y-18017), PPY12-OH, yGC4, or derivatives thereof.

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25. The method of claim 22, 23 or 24, wherein said yeast has been genetically engineered to expresses a heterologous protein.

- 26. A genetically engineered strain of a methylotrophic yeast, wherein
  - 5 said strain is transformed with at least one of the vectors of claims 1-21.
- 27. A method of reducing the glycosylation of a heterologous glycoprotein expressed from a methylotrophic yeast, comprising transforming cells of said methylotrophic yeast with at least one of the vectors of claims 1-21, and
  - 10 producing said glycoprotein from the transformed cells.
- 28. A method of producing a glycoprotein with reduced glycosylation in a methylotrophic yeast, comprising transforming cells of said methylotrophic yeast with at least one of the vectors of claims 1-21 and with a nucleotide sequence capable of expressing said glycoprotein in said yeast, and producing said glycoprotein from the transformed cells.

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- 29. A glycoprotein produced by the method of claim 27 or 28.
- 30. The glycoprotein of claim 29, wherein said glycoprotein has a reduced immunogenicity as relative to the glycoprotein produced from a wild type strain of said methylotrophic yeast.

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- 31. The glycoprotein of claim 29, wherein said glycoprotein is suitable
  - 25 for use in human therapeutics.
- 32. A kit comprising any of the vectors of claims 1-21.

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33. The kit of claim 32, further comprising a methylotrophic yeast

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strain.

34. A kit comprising the methylotrophic yeast strain of claim 26.

EcoR 1 (761) Xbal (824)

pGAPZalphaA 3147 bp

Xbal (1670)

MFmanase

5' AOX1

EcoR 1 (9688)

-AOX1TT

PIC9MFmanase

Amp R

9691 hp

COET-

ÀOX1 ∏

Zeodh CYC1 TT

XbaI (2475)

Eco RI/Xba I

1)PCR using 5'AOXI

3'AOX1

primer and

T4 DNA ligase

HDEL-Xbal primer 2) Eco RIIXba I

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LeoR I (1223) Avril (1229)

5'A0X1

Amp R

MFManHDEL

ORF

gAP.

EcoR 1 (1)

AOX1 ☐

PIC9 8023 Pp

SET I

**SAPZMFManHDEI** 

Ser. 1

4622 bp

3'AOX1

-Xba 1 (1539)

CYC1 TT /

FIGURE 2

27.2

pPIC9mMycManHDEL

Cloned via Xho I/Not1 in pPIC9

pPIC9mManHDEL

XhoI (3074)

ColE1

-A0X1 TT

PIC9MFManHDEI 9535 bp

Amp R-

ColE1

HIS 4

3'AOX1

FICURE 1

MFManHDEL

R

5'AOX1

Eco RI/Avr II

Eco RIIXba

P TÈF1

T4 DNA-ligase

and Manantisense 2)Xho I/Nof I 3)74 ligase Not I (546) Tho I (534) -A0X1 T 1)PCR using ManMycSense L929 (cycloheximide+ Tt induced) mouse cDNA library, Group 6, average insert length : 2000 bp.

nanaseHDEĽ

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GAP.

LSphi (1268) Xba I (1539)

pGAPZMFManHDEL

ColE1~ XbaI (298)

4622 bp

AOX1 TT

P EM7

Zeocin

CYC1 T

PCR product containing 3' cMyc tag

Sph I (17)

-P TEF1
-P EM7
-Zeocin

MFManMycHDEL ORF / pGAPZMF ManMycHDEL

4632 bp

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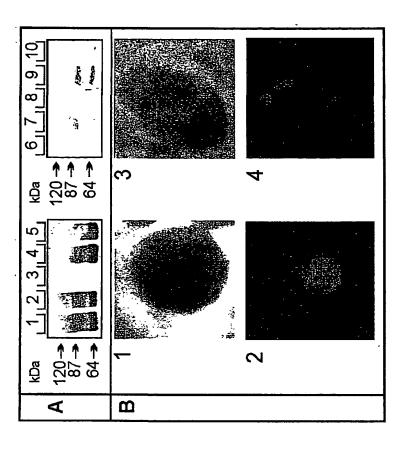


FIGURE 4

BsrBI (934) Xba I (1007)

pPICZ B 3330 bp

CYC1 TT

1)*Bst* Bl/Xba1 2)T4 DNA ligase AOX1 TT

P TEF1

Zeocin P EM7

5 AOXI

MFManMycHDEL ORF

pPICZB MFManMycHDEL 5080 bp

Col E1 -

FIGURE 3

AOX17

P EM7

Zeocin

CYC1 T

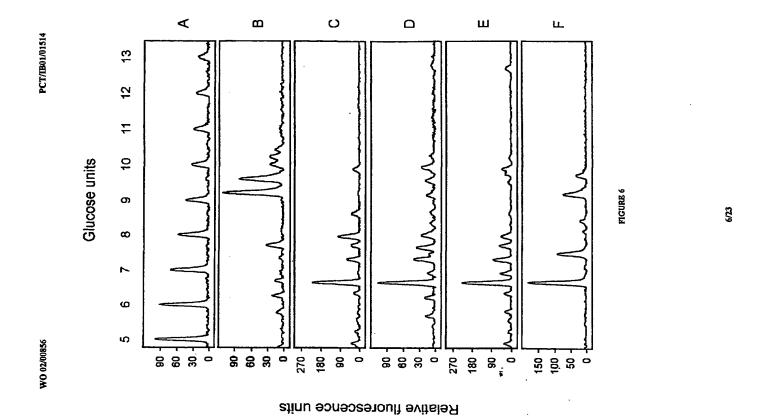
5' AOX1

CYC1 TT

GAP

Col E1 /

3/23



Mannosidase-Myc-HDEL

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OCH1-HA

PDI

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**PpURA3** 

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150C 112d 750

FIGURE 8

FIGURE 7

1100 1200 1300 1400 1500 1600 1700 1800 1900 2000 2100 2200 2300 2400 2500 2600 Glc, Glc, Glc,

NHA

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Scan

3600 1800

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## Pichia OCH1 Genomic fragment

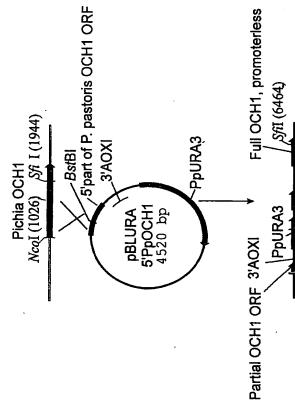


FIGURE 9

Ncol (1026) 1 Ncol (2884) Amp R

PICURE 10

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FIGURE 12

FIGURE 11

myc-tag/his6

Pin AI (2995)

AOX1 TT

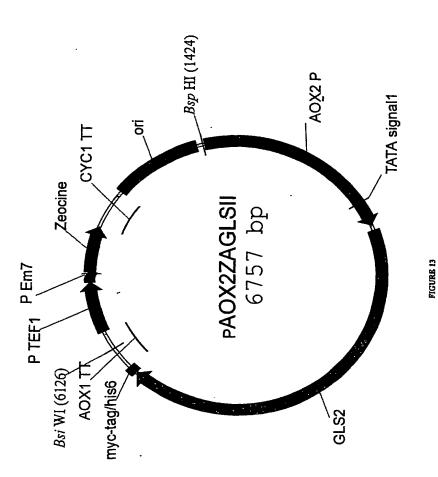
P TEF1

P EM7

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CilsII Pichia ex → ssion vector pAOX2Z(A)gls 12



GlsII Pichia es ession vector pPICZ(A)glsII

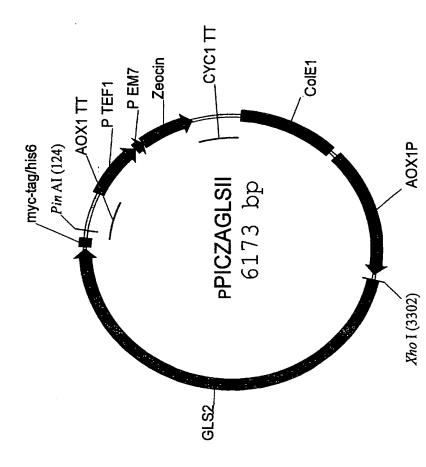


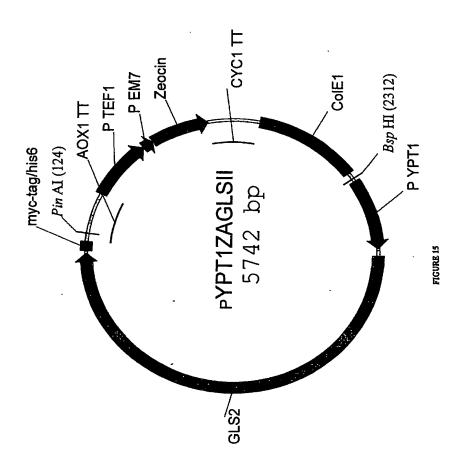
FIGURE 14

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GlsII Pichia ex\_ sssion vector pYPT1Z(A)glsI



GLSZ

GAP

GAP

GAP

Amp

PGAPADEIgISII

7236 bp

Myc-tag/his6

AOX1TT

FIGURE 16

# GIsII Pichia expression vector pAOX2ADE1glsII

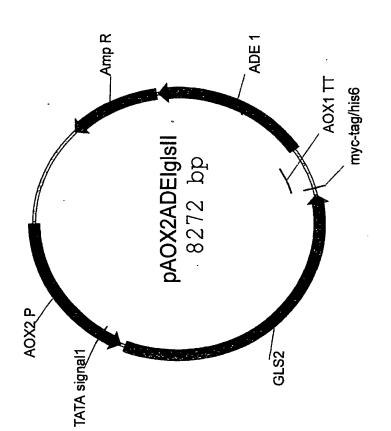


FIGURE 17

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# GISII Pichia expression vector pPICADE1gIsII

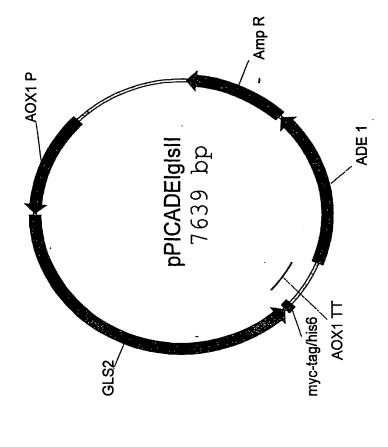


FIGURE 18

GISII Pichia expression vector pYPT1ADE1gIsII

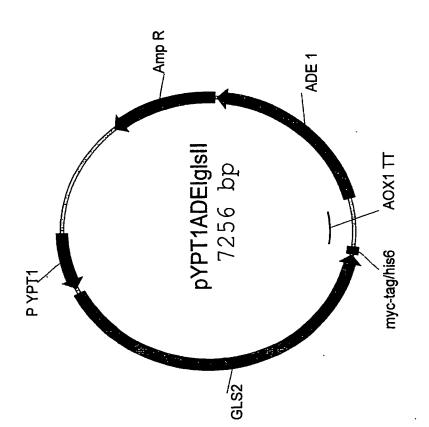


FIGURE 19

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GlsII Pichia expression vector pGAPZ(A)glsIIHDEL

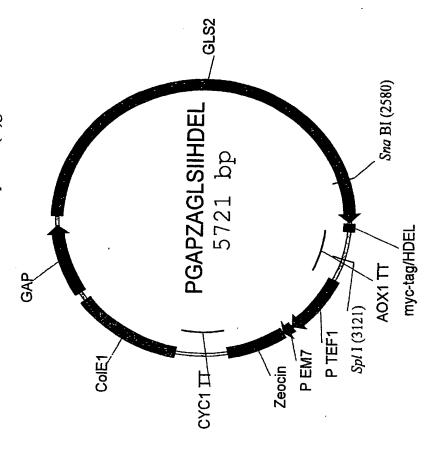
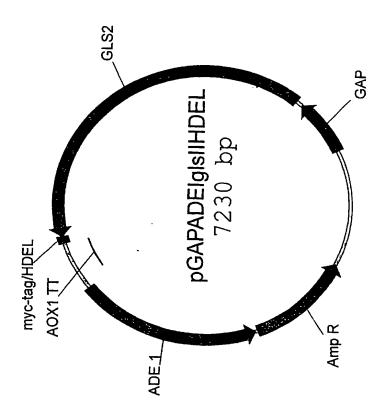


FIGURE 20

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GISII Pichia expression vector pGAPADE1gIsIIHDEL



PIGURE 21

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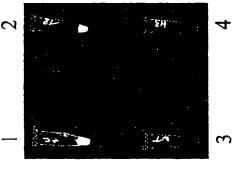
Glucosidase II assay on commercially available alpha-glucosidase



FIGURE 22

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Glucosidase II assay on heterologously expressed Pichia protein



PIGURE 23

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